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DESCRIPTION

METHOD OF NUCLEIC ACID INFUSION

TECHNICAL FIELD

The present invention relates to a novel method of nucleic acid infusion. More particularly, the present invention relates to a novel method of 5 nucleic acid infusion which utilizes difference in osmotic pressures.

BACKGROUND ART

The efficient infusion of oligonucleotide 10 such as antisense nucleic acids and small interfering RNA (siRNA) into cells is quite important for gene functional analysis that uses these molecules. Conventional methods of gene infusion include: a method of infusing into a cell a complex formed through an 15 ionic bond between a functional molecule and cell membrane negatively charged and a carrier positively charged such as a cationic liposome (Felgner et al., Proc. Natl. Acad. Sci. USA, 84, 7413 (1987); and Invitrogen, FOCUS, Vol. 21, No. 3 (1999)) or 20 polyethylenimine (Boussif et al., Proc. Natl. Acad. Sci. USA, 92, 7297 (1995)); a method of infusing a fusion molecule formed through a covalent bond between cell-permeable peptide and oligonucleotide (Thoren et al., FEBS Letters, 482, 265 (2000); and Nagahara et

al., *Nature medicine*, 4, 1449 (1998)); and a method of infusing a functional molecule into a hole that is transiently made on a cell by the application of electric pulses (Neumann et al., *EMBO J*, 1, 841
5 (1982)).

The method of infusion that uses a complex with a positively charged carrier generally has the favorable efficiency of infusion into adherent culture cell lines. However, the method uses uptake mechanisms
10 intrinsic to cells in the cellular uptake of the oligonucleotide-carrier complex from the outside to the inside and therefore has a difficulty with infusion into cells having low uptake activity. Moreover, the carrier itself has cytotoxicity and immunoadjuvant
15 activity and is therefore difficult to infuse into floating cells such as lymphocytes and primary cultured cells. The method of infusing a fusion molecule formed through a covalent bond between cell-permeable peptide and oligonucleotide presents problems with the reaction
20 efficiency of covalent bond formation and the complicated reaction and also presents problems in that the method observes not the action of the oligonucleotide alone but the overall action of the molecule formed through the covalent bond between the
25 cell-permeable peptide and the functional molecule. Alternatively, the method of infusing a functional molecule into a hole that is transiently made on a cell by the application of electric pulses presents problems

in that cells suffer severe damage due to the electric pulses, resulting in the disruption of most of the cells. Thus, each of the conventional methods of nucleic acid infusion has problems. Under these 5 circumstances, there has been a demand for a novel method of nucleic acid infusion that is capable of efficiently infusing a nucleic acid, regardless of cell types.

10 DISCLOSURE OF THE INVENTION

An object of the present invention is to provide a novel method of nucleic acid infusion. More particularly, an object of the present invention is to provide a novel method of nucleic acid infusion that 15 utilizes osmotic pressure difference.

The present inventors have diligently studied for attaining the above-described objects. As a result, the present inventors have successfully developed a method of efficient nucleic acid infusion 20 into a cell, which is independent of carriers, cell-permeable peptide and electric pulses. That is, the present invention allows the efficient infusion of a nucleic acid such as oligonucleotide into a cell by utilizing osmotic pressure difference between an 25 extracellular fluid and an intracellular fluid to promote pinocytosis that is known as one of phenomena in which cells take up extracellular solutions.

The infusion of low molecular weight

compounds and proteins into cells by use of pinocytosis has previously been reported (Craig Y Okada et al., Cell, 29, 33-41, 1982). However, recently, this technique has already been rendered obsolete and has
5 not been reported to be used in nucleic acid infusion.

The present inventors have investigated a method of nucleic acid infusion that utilizes osmotic pressure difference. That is, the present inventors have diligently conducted studies on a method of
10 nucleic acid infusion by (1) bringing a hypertonic solution containing a nucleic acid and cells into contact with each other to induce the cellular uptake of the nucleic acid by pinocytosis and (2) then lowering the osmotic pressure of the above-described
15 hypertonic solution to induce the disruption of pinocytic vesicles in the cells and the release of the nucleic acid into the cells. As a result, surprisingly, the present inventors have found that the use of the method of the present invention allows
20 nucleic acid infusion with higher efficiency than ever. In addition, the present inventors have found that the method of the present invention allows efficient oligonucleotide infusion into cell types that are hardly infused with a nucleic acid by conventional
25 techniques. Since the method of the present invention does not employ adjuvants (such as liposomes and polyethylenimine) conventionally used, the method overcomes the problems of conventional methods such as

cytotoxicity and immunoadjuvant activity. Therefore, the present invention makes it possible to efficiently infuse oligonucleotide into not only adherent cell lines but also primary cultured cells and lymphocytes
5 that are highly sensitive to cytotoxicity.

The present invention has been completed on the basis of such findings.

Namely, the present invention is as follows:

- (1) a method of nucleic acid infusion, comprising the
10 following steps (a) and (b):

(a) bringing a nucleic acid, a hypertonic solution and cells into contact with each other; and

(b) lowering osmotic pressure of the hypertonic solution after the above-described step (a);
15 (2) the method of nucleic acid infusion according to the above-described (1), wherein the above-described step (b) comprises lowering the osmotic pressure by bringing a hypotonic solution and the cells into contact with each other;
20 (3) the method of nucleic acid infusion according to the above-described (1) or (2), wherein the nucleic acid is oligonucleotide;

(4) the method of nucleic acid infusion according to the above-described (3), wherein the oligonucleotide is
25 single-stranded oligonucleotide, double-stranded oligonucleotide or an analog thereof;

(5) the method of nucleic acid infusion according to the above-described (3) or (4), wherein the

oligonucleotide is deoxyribonucleotide (DNA),
ribonucleotide (RNA), phosphorothioate
oligodeoxynucleotide, a 2'-O-(2-methoxy)ethyl-modified
nucleic acid (2'-MOE-modified nucleic acid), small
5 interfering RNA (siRNA), a locked nucleic acid (LNA), a
peptide nucleic acid (PNA) or morpholino antisense
oligonucleotide;

(6) the method of nucleic acid infusion according to
any of the above-described (1) to (5), wherein the
10 hypertonic solution comprises at least one substance
which is oligosaccharide or polyhydric alcohol;

(7) the method of nucleic acid infusion according to
the above-described (6), wherein the oligosaccharide is
disaccharide;

15 (8) the method of nucleic acid infusion according to
the above-described (7), wherein the disaccharide is
sucrose, maltose or lactose;

(9) the method of nucleic acid infusion according to
the above-described (6), wherein the polyhydric alcohol
20 is diol, triol, polyol or sugar alcohol;

(10) the method of nucleic acid infusion according to
the above-described (9), wherein the diol is a glycol
derivative;

(11) the method of nucleic acid infusion according to
25 the above-described (9), wherein the triol is a
glycerol derivative;

(12) the method of nucleic acid infusion according to
the above-described (9), wherein the polyol is a

polyglycol derivative;

(13) the method of nucleic acid infusion according to the above-described (9), wherein the sugar alcohol is monosaccharide alcohol or oligosaccharide alcohol;

5 (14) the method of nucleic acid infusion according to the above-described (13), wherein the oligosaccharide alcohol is disaccharide alcohol;

(15) the method of nucleic acid infusion according to the above-described (10), wherein the glycol derivative
10 is ethylene glycol;

(16) the method of nucleic acid infusion according to the above-described (11), wherein the glycerol derivative is glycerol;

(17) the method of nucleic acid infusion according to
15 the above-described (12), wherein the polyglycol derivative is polyethylene glycol;

(18) the method of nucleic acid infusion according to the above-described (17), wherein the polyethylene glycol has a molecular weight of 2000 or less;

20 (19) the method of nucleic acid infusion according to the above-described (18), wherein the polyethylene glycol is PEG200, PEG300, PEG400, PEG600, PEG1000, PEG1500, PEG1540 or PEG 2000;

(20) the method of nucleic acid infusion according to
25 the above-described (13), wherein the monosaccharide alcohol is mannitol, sorbitol, xylitol or erythritol;

(21) the method of nucleic acid infusion according to the above-described (14), wherein the disaccharide

- alcohol is maltitol, lactitol or reduced palatinose;
- (22) the method of nucleic acid infusion according to the above-described (6), wherein the hypertonic solution comprises (a) at least one substance which is
- 5 oligosaccharide or sugar alcohol and (b) at least one substance which is diol, triol or polyol;
- (23) the method of nucleic acid infusion according to the above-described (22), wherein the oligosaccharide is disaccharide;
- 10 (24) the method of nucleic acid infusion according to the above-described (23), wherein the disaccharide is sucrose, maltose or lactose;
- (25) the method of nucleic acid infusion according to the above-described (22), wherein the sugar alcohol is
- 15 monosaccharide alcohol or oligosaccharide alcohol;
- (26) the method of nucleic acid infusion according to the above-described (25), wherein the oligosaccharide alcohol is disaccharide alcohol;
- (27) the method of nucleic acid infusion according to
- 20 the above-described (25), wherein the monosaccharide alcohol is mannitol, sorbitol, xylitol or erythritol;
- (28) the method of nucleic acid infusion according to the above-described (26), wherein the disaccharide alcohol is maltitol, lactitol or reduced palatinose;
- 25 (29) the method of nucleic acid infusion according to the above-described (22), wherein the diol is a glycol derivative;
- (30) the method of nucleic acid infusion according to

the above-described (22), wherein the triol is a glycerol derivative;

(31) the method of nucleic acid infusion according to the above-described (22), wherein the polyol is a
5 polyglycol derivative;

(32) the method of nucleic acid infusion according to the above-described (29), wherein the glycol derivative is ethylene glycol;

(33) the method of nucleic acid infusion according to
10 the above-described (30), wherein the glycerol derivative is glycerol;

(34) the method of nucleic acid infusion according to the above-described (31), wherein the polyglycol derivative is polyethylene glycol;

15 (35) the method of nucleic acid infusion according to the above-described (34), wherein the polyethylene glycol has a molecular weight of 2000 or less;

(36) the method of nucleic acid infusion according to the above-described (35), wherein the polyethylene
20 glycol is PEG200, PEG300, PEG400, PEG600, PEG1000, PEG1500, PEG1540 or PEG2000;

(37) the method of nucleic acid infusion according to the above-described (22), wherein the hypertonic solution comprises (a) at least one substance selected
25 from sucrose, mannitol, sorbitol and xylitol and (b) at least one substance selected from PEG200, PEG300, PEG400, PEG600, PEG1000, PEG1500, PEG1540, PEG2000 and glycerol;

- (38) the method of nucleic acid infusion according to the above-described (37), wherein the hypertonic solution comprises (a) sucrose, mannitol, sorbitol or xylitol and (b) PEG200, PEG300, PEG400, PEG600,
5 PEG1000, PEG1500, PEG1540, PEG2000 or glycerol;
- (39) the method of nucleic acid infusion according to the above-described (6), wherein the hypertonic solution comprises one substance which is oligosaccharide or sugar alcohol;
- 10 (40) the method of nucleic acid infusion according to the above-described (39), wherein the oligosaccharide is disaccharide;
- (41) the method of nucleic acid infusion according to the above-described (40), wherein the disaccharide is sucrose, maltose or lactose;
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- (42) the method of nucleic acid infusion according to the above-described (39), wherein the sugar alcohol is monosaccharide alcohol or oligosaccharide alcohol;
- (43) the method of nucleic acid infusion according to
20 the above-described (42), wherein the oligosaccharide alcohol is disaccharide alcohol;
- (44) the method of nucleic acid infusion according to the above-described (42), wherein the monosaccharide alcohol is mannitol, sorbitol, xylitol or erythritol;
- 25 (45) the method of nucleic acid infusion according to the above-described (43), wherein the disaccharide alcohol is maltitol, lactitol or reduced palatinose;
- (46) the method of nucleic acid infusion according to

the above-described (39), wherein the hypertonic solution comprises sucrose, mannitol, sorbitol, xylitol or maltitol;

(47) the method of nucleic acid infusion according to
5 any of the above-described (1) to (46), wherein the oligosaccharide and/or the polyhydric alcohol present in the hypertonic solution has a total molarity of 0.29 M to 3 M;

(48) the method of nucleic acid infusion according to
10 the above-described (47), wherein the oligosaccharide and/or the polyhydric alcohol present in the hypertonic solution has a total molarity of 0.5 M to 2.1 M;

(49) the method of nucleic acid infusion according to any of the above-described (2) to (48), wherein the
15 hypotonic solution has isotonic osmotic pressure or lower;

(50) a reagent for nucleic acid infusion, comprising as an ingredient at least one substance which is oligosaccharide or polyhydric alcohol;

20 (51) the reagent for nucleic acid infusion according to the above-described (50), wherein the reagent is used in a method of nucleic acid infusion according to any of the above-described (1) to (49);

(52) the reagent for nucleic acid infusion according to
25 the above-described (50) or (51), wherein the oligosaccharide is disaccharide;

(53) the reagent for nucleic acid infusion according to the above-described (52), wherein the disaccharide is

sucrose, maltose or lactose;

(54) the reagent for nucleic acid infusion according to the above-described (50) or (51), wherein the polyhydric alcohol is diol, triol, polyol or sugar

5 alcohol;

(55) the reagent for nucleic acid infusion according to the above-described (54), wherein the diol is a glycol derivative;

10 (56) the reagent for nucleic acid infusion according to the above-described (54), wherein the triol is a glycerol derivative;

(57) the reagent for nucleic acid infusion according to the above-described (54), wherein the polyol is a polyglycol derivative;

15 (58) the reagent for nucleic acid infusion according to the above-described (54), wherein the sugar alcohol is monosaccharide alcohol or oligosaccharide alcohol;

(59) the reagent for nucleic acid infusion according to the above-described (58), wherein the oligosaccharide 20 alcohol is disaccharide alcohol;

(60) the reagent for nucleic acid infusion according to the above-described (55), wherein the glycol derivative is ethylene glycol;

(61) the reagent for nucleic acid infusion according to 25 the above-described (56), wherein the glycerol derivative is glycerol;

(62) the reagent for nucleic acid infusion according to the above-described (57), wherein the polyglycol

derivative is polyethylene glycol;

(63) the reagent for nucleic acid infusion according to the above-described (62), wherein the polyethylene glycol has a molecular weight of 2000 or less;

5 (64) the reagent for nucleic acid infusion according to the above-described (63), wherein the polyethylene glycol is PEG200, PEG300, PEG400, PEG600, PEG1000, PEG1500, PEG1540 or PEG2000;

(65) the reagent for nucleic acid infusion according to
10 the above-described (58), wherein the monosaccharide alcohol is mannitol, sorbitol, xylitol or erythritol;

(66) the reagent for nucleic acid infusion according to the above-described (59), wherein the disaccharide alcohol is maltitol, lactitol or reduced palatinose;

15 (67) the reagent for nucleic acid infusion according to the above-described (50) or (51), wherein the reagent comprises as ingredients (a) at least one substance which is oligosaccharide or sugar alcohol and (b) at least one substance which is diol, triol or polyol;

20 (68) the reagent for nucleic acid infusion according to the above-described (67), wherein the oligosaccharide is disaccharide;

(69) the reagent for nucleic acid infusion according to the above-described (68), wherein the disaccharide is
25 sucrose, maltose or lactose;

(70) the reagent for nucleic acid infusion according to the above-described (67), wherein the sugar alcohol is monosaccharide alcohol or oligosaccharide alcohol;

- (71) the reagent for nucleic acid infusion according to the above-described (70), wherein the oligosaccharide alcohol is disaccharide alcohol;
- (72) the reagent for nucleic acid infusion according to 5 the above-described (70), wherein the monosaccharide alcohol is mannitol, sorbitol, xylitol or erythritol;
- (73) the reagent for nucleic acid infusion according to the above-described (71), wherein the disaccharide alcohol is maltitol, lactitol or reduced palatinose;
- 10 (74) the reagent for nucleic acid infusion according to the above-described (67), wherein the diol is a glycol derivative;
- (75) the reagent for nucleic acid infusion according to the above-described (67), wherein the triol is a 15 glycerol derivative;
- (76) the reagent for nucleic acid infusion according to the above-described (67), wherein the polyol is a polyglycol derivative;
- (77) the reagent for nucleic acid infusion according to 20 the above-described (74), wherein the glycol derivative is ethylene glycol;
- (78) the reagent for nucleic acid infusion according to the above-described (75), wherein the glycerol derivative is glycerol;
- 25 (79) the reagent for nucleic acid infusion according to the above-described (76), wherein the polyglycol derivative is polyethylene glycol;
- (80) the reagent for nucleic acid infusion according to

the above-described (79), wherein the polyethylene glycol has a molecular weight of 2000 or less;

(81) the reagent for nucleic acid infusion according to the above-described (80), wherein the polyethylene

5 glycol is PEG200, PEG300, PEG400, PEG600, PEG1000, PEG1500, PEG1540 or PEG2000;

(82) the reagent for nucleic acid infusion according to the above-described (67), wherein the reagent comprises as ingredients (a) at least one substance selected from

10 sucrose, mannitol, sorbitol and xylitol and (b) at least one substance selected from PEG200, PEG300, PEG400, PEG600, PEG1000, PEG1500, PEG1540, PEG2000 and glycerol;

(83) the reagent for nucleic acid infusion according to

15 the above-described (82), wherein the reagent comprises as ingredients (a) sucrose, mannitol, sorbitol or xylitol and (b) PEG200, PEG300, PEG400, PEG600, PEG1000, PEG1500, PEG1540, PEG2000 or glycerol;

(84) the reagent for nucleic acid infusion according to

20 the above-described (50) or (51), wherein the reagent comprises as an ingredient at least one substance which is oligosaccharide or sugar alcohol;

(85) the reagent for nucleic acid infusion according to the above-described (84), wherein the oligosaccharide

25 is disaccharide;

(86) the reagent for nucleic acid infusion according to the above-described (85), wherein the disaccharide is

sucrose, maltose or lactose;

- (87) the reagent for nucleic acid infusion according to the above-described (84), wherein the sugar alcohol is monosaccharide alcohol or oligosaccharide alcohol;
- (88) the reagent for nucleic acid infusion according to 5 the above-described (87), wherein the oligosaccharide alcohol is disaccharide alcohol;
- (89) the reagent for nucleic acid infusion according to the above-described (87), wherein the monosaccharide alcohol is mannitol, sorbitol, xylitol or erythritol;
- 10 (90) the reagent for nucleic acid infusion according to the above-described (88), wherein the disaccharide alcohol is maltitol, lactitol or reduced palatinose;
- (91) the reagent for nucleic acid infusion according to the above-described (84), wherein the reagent comprises 15 as an ingredient sucrose, mannitol, sorbitol, xylitol or maltitol;
- (92) the reagent for nucleic acid infusion according to any of the above-described (50) to (91), wherein the ingredient is in the form of a solid or liquid;
- 20 (93) the reagent for nucleic acid infusion according to the above-described (92), wherein when the reagent comprises two or more ingredients which are each oligosaccharide or polyhydric alcohol, and the ingredients are each independently in the form of a 25 solid or liquid;
- (94) the reagent for nucleic acid infusion according to any of the above-described (50) to (93), wherein the reagent is prepared in advance so that the

oligosaccharide and/or the polyhydric alcohol in a hypertonic solution has a total molarity of 0.29 M to 3 M;

(95) the reagent for nucleic acid infusion according to 5 the above-described (94), wherein the reagent is prepared in advance so that the oligosaccharide and/or the polyhydric alcohol in the hypertonic solution has a total molarity of 0.5 M to 2.1 M;

(96) a kit for nucleic acid infusion, comprising a 10 reagent according to any of the above-described (50) to (95);

(97) use of a reagent or a kit according to any of the above-described (50) to (96) in nucleic acid infusion;

(98) a nucleic acid infusion agent, comprising as an 15 ingredient a reagent or a kit according to any of the above-described (50) to (96);

(99) a cell with a nucleic acid infused thereinto by a method of infusion according to any of the above-described (1) to (49);

20 (100) a macrophage-like cell line RAW264.7 cell, a hybridoma D011.10 cell, a primary cultured hepatocyte, a primary cultured myoblast, a primary cultured muscle cell, a primary cultured adipocyte precursor cell, a primary cultured adipocyte or a primary cultured neuron 25 with a nucleic acid infused thereinto by a method of infusion according to any of the above-described (1) to (49);

(101) Cell-derived RNA or protein prepared from a cell

according to the above-described (99) or (100); and
(102) a method of functional analysis of a gene or
protein associated with an infused nucleic acid,
characterized by using a cell according to the above-
5 described (99) or (100) or RNA or protein according to
the above-described (101).

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a result of oligonucleotide
10 infusion into murine macrophage-like cell line RAW264.7
cells by a method of nucleic acid infusion of the
present invention (upper column), an Oligofectamine
method (middle column) and a polyethylenimine method
(lower column). In the drawing, the left and right
15 columns show the bright-field and fluorescence images,
respectively, of the infused cells;

FIG. 2 shows a result of measuring siRNA
effect by quantitative PCR after TLR4 siRNA infusion
into RAW264.7 cells by the method of nucleic acid
20 infusion of the present invention. In the drawing,
TLR4 siRNA indicates a result of TLR4 siRNA infusion,
and Negative Control siRNA indicates a result of
infusion of siRNA that serves as a negative control.
The vertical axis indicates the relative amount of TLR4
25 mRNA after normalization with the amount of GAPDH mRNA;

FIG. 3 shows a result of measuring the
inhibitory effect of stimulation with LPS or GLA-60 on
TNF α production after TLR4 siRNA infusion into RAW264.7

cells by the method of nucleic acid infusion of the present invention. In the drawing, TLR4 siRNA indicates a result of TLR4 siRNA infusion, and Negative Control siRNA indicates a result of infusion of siRNA
5 that serves as a negative control. The vertical axis indicates the amount of TNF α produced;

FIG. 4 shows a result of measuring infusion efficiency (upper diagram) and cytotoxicity (lower diagram) after FITC-labeled oligonucleotide infusion
10 into RAW264.7 cells by the method of nucleic acid infusion of the present invention. The horizontal axis indicates the amount of fluorescence emitted from FITC as a marker for infusion efficiency (upper diagram) and the amount of fluorescence emitted from 7AAD as a
15 marker for cytotoxicity (lower diagram), and the vertical axis indicates the number of events;

FIG. 5 shows a result of using RAW264.7 cells to measure the effect of types of oligosaccharide and polyhydric alcohol contained in a hypertonic solution
20 on siRNA infusion into cells. In the drawing, GAPDH siRNA indicates a result of GAPDH siRNA infusion, and Negative Control siRNA indicates a result of infusion of siRNA that serves as a negative control. The vertical axis indicates the relative amount of GAPDH
25 mRNA after normalization with the amount of cyclophilin mRNA;

FIG. 6 shows a result of using RAW264.7 cells to measure the effect of a combination of sucrose and

polyhydric alcohol (PEG) contained in a hypertonic solution on siRNA infusion into cells. In the drawing, GAPDH siRNA indicates a result of GAPDH siRNA infusion, and Negative Control siRNA indicates a result of

5 infusion of siRNA that serves as a negative control.

The vertical axis indicates the relative amount of GAPDH mRNA after normalization with the amount of cyclophilin mRNA;

FIG. 7 shows a result of using RAW264.7 cells
10 to measure the effect of a combination of sorbitol and polyhydric alcohol (PEG) contained in a hypertonic solution on siRNA infusion into cells. In the drawing, GAPDH siRNA indicates a result of GAPDH siRNA infusion, and Negative Control siRNA indicates a result of

15 infusion of siRNA that serves as a negative control.

The vertical axis indicates the relative amount of GAPDH mRNA after normalization with the amount of cyclophilin mRNA;

FIG. 8 shows a result of using DO11.10 cells
20 to investigate whether the method of nucleic acid infusion of the present invention can also be applied to a nucleic acid with no electric charge. The upper diagram indicates a result of infusion of morpholino antisense oligonucleotide that is a nucleic acid analog
25 with no electric charge, and the lower diagram indicates a result of infusion of phosphorothioate oligodeoxynucleotide that is a nucleic acid analog with an electric charge. In the upper diagram, the

horizontal axis indicates the amount of fluorescence emitted from fluorescein as a marker for infusion, and the vertical axis indicates the number of events. In the lower diagram, the horizontal axis indicates the 5 amount of fluorescence emitted from FITC as a maker for infusion, and the vertical axis indicates the number of events;

FIG. 9 shows a result of using D011.10 cells to examine the effect of siRNA repetitive infusion by 10 the method of nucleic acid infusion of the present invention. In the drawing, GAPDH siRNA indicates a result of GAPDH siRNA infusion, and Negative Control siRNA indicates a result of infusion of siRNA that serves as a negative control. The vertical axis 15 indicates the relative amount of GAPDH mRNA after normalization with the amount of cyclophilin mRNA;

FIG. 10 shows a result of measuring infusion efficiency (knockdown activity) after siRNA infusion into primary cultured differentiated rat adipocytes by 20 the method of nucleic acid infusion of the present invention. In the drawing, GAPDH siRNA indicates a result of GAPDH siRNA infusion, and Negative Control siRNA indicates a result of infusion of siRNA that serves as a negative control. The vertical axis 25 indicates the relative amount of GAPDH mRNA after normalization with the amount of cyclophilin mRNA; and

FIG. 11 shows a result of measuring infusion efficiency (knockdown activity) at a protein level

after siRNA repetitive infusion into RAW264.7 cells by the method of nucleic acid infusion of the present invention. In the drawing, Ctrl indicates negative control siRNA, and TLR2 indicates siRNA for TLR2.

- 5 Anti-TLR2 indicates a TLR2 protein detected with an anti-TLR2 antibody, and Anti-GAPDH indicates a GAPDH protein detected with an anti-GAPDH antibody.

BEST MODE FOR CARRYING OUT THE INVENTION

10 The present invention provides a method of nucleic acid infusion, comprising at least the following steps (a) and (b):
(a) bringing a nucleic acid, a hypertonic solution and cells into contact with each other; and
15 (b) lowering osmotic pressure of the hypertonic solution after the above-described step (a).

The nucleic acid used in the step (a) is not limited by types in terms of the principle of the method, and any nucleic acid can be used as the nucleic 20 acid to be infused. That is, the nucleic acid may be any of oligonucleotide and polynucleotide and may assume any morphology of single and double strands and an analog thereof. When the nucleic acid of the present invention is single-stranded, any of sense and 25 antisense strands can be used. When the nucleic acid of the present invention is polynucleotide, the nucleic acid may assume the morphology of a plasmid.

Preferred examples of the nucleic acid of the

present invention can include oligonucleotide.

Oligonucleotide to be infused is not limited by types in terms of the principle of the method, and any of single-stranded oligonucleotide, double-stranded

5 oligonucleotide and an analog thereof can be used.

Specifically, the oligonucleotide can be exemplified by deoxyribonucleotide (DNA), ribonucleotide (RNA), phosphorothioate oligodeoxynucleotide (see Example 1), a 2'-O-(2-methoxy)ethyl-modified nucleic acid (2'-MOE-
10 modified nucleic acid), small interfering RNA (siRNA; see Examples 2 and 3), a locked nucleic acid (LNA; Singh et al., Chem. Commun., 455, 1998), a peptide nucleic acid (PNA; Nielsen et al., Science, 254, 1497, 1991), or morpholino antisense oligonucleotide
15 (Summerton and Weller, Antisense & Nucleic Acid Drug Development, 7, 187, 1997).

The concentration of the nucleic acid of the present invention in the hypertonic solution may be any concentration at which the nucleic acid can be infused
20 into cells. The concentration can include usually a range of 0.01 μ M to 1 mM, preferably 1 μ M to 100 μ M.

The hypertonic solution of the present invention that can be used in the step (a) may be any solution that maintains isotonic osmotic pressure or
25 higher osmotic pressure and allows the cellular uptake of the nucleic acid by bringing the hypertonic solution containing the nucleic acid and cells into contact with each other. Preferred examples of the hypertonic

solution include a hypertonic solution comprising at least one substance which is oligosaccharide or polyhydric alcohol.

In this context, concrete examples of the 5 oligosaccharide include disaccharide, trisaccharide, tetrasaccharide, pentasaccharide or decasaccharide, with disaccharide preferred. The disaccharide includes sucrose, maltose, lactose, trehalose, levanbiose, chitobiose, vicianose, sambubiose, melibiose, 10 epicellobiose, turanose, rutinose or robinobiose, with sucrose, maltose or lactose preferred.

Concrete examples of the above-described polyhydric alcohol include diol, triol, polyol or sugar alcohol, with triol, polyol or sugar alcohol more 15 preferred.

In this context, the diol includes a glycol derivative. The glycol derivative is exemplified by ethylene glycol.

The triol includes a glycerol derivative. 20 The glycerol derivative includes glycerol.

The polyol includes a polyglycol derivative. The polyglycol derivative includes polyethylene glycol.

The polyethylene glycol may have any molecular weight not greater than 2000. Specifically, 25 the polyethylene glycol is exemplified by PEG200, PEG300, PEG400, PEG600, PEG1000, PEG1500, PEG1540 and PEG2000.

The above-described sugar alcohol includes

monosaccharide alcohol or oligosaccharide alcohol.

The monosaccharide alcohol includes mannitol, sorbitol, xylitol or erythritol.

The above-described oligosaccharide alcohol
5 includes disaccharide alcohol. The disaccharide alcohol includes maltitol, lactitol or reduced palatinose.

The hypertonic solution used in the method of nucleic acid infusion of the present invention may
10 comprise at least one substance which is a variety of oligosaccharides or polyhydric alcohols described above. Specifically, the hypertonic solution may comprise at least one substance arbitrarily selected from among the substances listed above, for example,
15 sucrose, maltose, lactose, trehalose, levanbiose, chitobiose, vicianose, sambubiose, melibiose, epicellobiose, turanose, rutinose, robinobiose, ethylene glycol, glycerol, polyethylene glycol (PEG having a molecular weight of 2000 or less, e.g.,
20 PEG200, PEG300, PEG400, PEG600, PEG1000, PEG1500, PEG1540 and PEG2000), mannitol, sorbitol, xylitol, erythritol, maltitol, lactitol and reduced palatinose.
To be more specific, the hypertonic solution is exemplified by a hypertonic solution comprising one
25 substance, two substances, three substances or four or more substances arbitrarily selected from among the substances listed above. In this case, it is necessary to prepare the hypertonic solution so that the

oligosaccharide and/or the polyhydric alcohol present in the hypertonic solution has a total molarity of 0.29 M to 3 M for allowing the cellular uptake of the nucleic acid when the hypertonic solution, the nucleic acid and cells are brought into contact with each other. The total molarity may be any molarity that falls within this range and includes a range of preferably 0.35 M to 2.5 M, more preferably 0.35 M to 2.1 M, even more preferably 0.5 M to 2.1 M.

10 Although suitable molarity varies depending on cells, molarity suitable for a desired cell can readily be determined, for example, by using three concentrations of 1 M, 1.5 M and 2 M to investigate infusion into the cell.

15 The hypertonic solution used in the method of nucleic acid infusion of the present invention may comprise at least one substance which is oligosaccharide or polyhydric alcohol, as described above. Alternatively, the hypertonic solution may 20 comprise only one substance or two or more substances which are each oligosaccharide or polyhydric alcohol. When the hypertonic solution comprises two or more substances which are each oligosaccharide or polyhydric alcohol, it is preferred that the hypertonic solution 25 should comprise (1) at least one substance which is oligosaccharide or sugar alcohol and (2) at least one substance which is diol, triol or polyol.

In this context, concrete examples of the

oligosaccharide used include disaccharide, trisaccharide, tetrasaccharide, pentasaccharide or decasaccharide, with disaccharide preferred. The disaccharide includes sucrose, maltose, lactose, 5 trehalose, levanbiose, chitobiose, vicianose, sambubiose, melibiose, epicelllobiose, turanose, rutinose or robinobiose, with sucrose, maltose or lactose preferred.

The above-described sugar alcohol includes 10 monosaccharide alcohol or oligosaccharide alcohol.

The monosaccharide alcohol includes mannitol, sorbitol, xylitol or erythritol.

The above-described oligosaccharide alcohol includes disaccharide alcohol.

15 The disaccharide alcohol includes maltitol, lactitol or reduced palatinose.

The above-described diol includes a glycol derivative. The glycol derivative includes ethylene glycol.

20 The triol includes a glycerol derivative. The glycerol derivative includes glycerol.

The polyol includes a polyglycol derivative. The polyglycol derivative includes polyethylene glycol.

25 The polyethylene glycol may have any molecular weight not greater than 2000. Specifically, the polyethylene glycol is exemplified by PEG200, PEG300, PEG400, PEG600, PEG1000, PEG1500, PEG1540 and PEG2000.

A preferred combination of (1) at least one substance which is oligosaccharide or sugar alcohol and (2) at least one substance which is diol, triol or polyol contained in the hypertonic solution of the 5 present invention as described above is exemplified by a combination between (1) at least one substance selected from sucrose, mannitol, sorbitol and xylitol and (2) at least one substance selected from PEG200, PEG300, PEG400, PEG600, PEG1000, PEG1500, PEG1540, 10 PEG2000 and glycerol.

More preferably, the hypertonic solution comprising the combination is exemplified by a hypertonic solution comprising (1) one substance which is oligosaccharide or sugar alcohol and (2) one 15 substance which is diol, triol or polyol and specifically exemplified by a hypertonic solution comprising (1) sucrose, mannitol, sorbitol or xylitol and (2) PEG200, PEG300, PEG400, PEG600, PEG1000, PEG1500, PEG1540, PEG2000 or glycerol.

Such a hypertonic solution of the present invention comprising two or more substances which are each oligosaccharide or polyhydric alcohol may have each ingredient at any molarity, as long as the hypertonic solution is prepared so that the 20 oligosaccharide and/or the polyhydric alcohol present in the hypertonic solution has a total molarity of 0.29 M to 3 M. The total molarity includes a range of 25 preferably 0.35 M to 2.5 M, more preferably 0.35 M to

2.1 M, even more preferably 0.5 M to 2.1 M.

Although suitable molarity varies depending on cells, molarity suitable for a desired cell can readily be determined; for example, by using three 5 concentrations of 1 M, 1.5 M and 2 M to investigate infusion into the cell.

When the hypertonic solution of the present invention comprises two substances which are each oligosaccharide or polyhydric alcohol, the molarity of 10 each ingredient may be any value as described above, as long as total molarity of the two substances falls within the above-described range. In this case, the molarity of (1) one substance which is oligosaccharide or sugar alcohol can be exemplified by a range of 0.5 M 15 to 2 M, and the molarity of (2) one substance which is diol, triol or polyol can be exemplified by 0.1 M.

When the hypertonic solution used in the method of nucleic acid infusion of the present invention comprises only one substance which is 20 oligosaccharide or polyhydric alcohol, it is preferred to select the one substance from oligosaccharide or sugar alcohol.

In this context, concrete examples of the oligosaccharide used include disaccharide, 25 trisaccharide, tetrasaccharide, pentasaccharide or decasaccharide, with disaccharide preferred. The disaccharide includes sucrose, maltose, lactose, trehalose, levanbiose, chitobiose, vicianose,

sambubiose, melibiose, epicellobiose, turanose, rutinose or robinobiose, with sucrose, maltose or lactose preferred.

The above-described sugar alcohol includes
5 monosaccharide alcohol or oligosaccharide alcohol.

The monosaccharide alcohol includes mannitol, sorbitol, xylitol or erythritol.

The above-described oligosaccharide alcohol includes disaccharide alcohol.

10 The disaccharide alcohol includes maltitol, lactitol or reduced palatinose.

The hypertonic solution of the present invention comprising only one substance which is oligosaccharide or sugar alcohol as described above is
15 preferably a hypertonic solution comprising sucrose, mannitol, sorbitol, xylitol or maltitol.

Such a hypertonic solution of the present invention comprising only one substance which is oligosaccharide or polyhydric alcohol may be prepared
20 so that the oligosaccharide or the polyhydric alcohol present in the hypertonic solution has a molarity of 0.29 M to 3 M. The molarity includes a range of preferably 0.35 M to 2.5 M, more preferably 0.35 M to 2.1 M, even more preferably 0.5 M to 2.1 M.

25 Although suitable molarity varies depending on cells, molarity suitable for a desired cell can readily be determined, for example, by using three concentrations of 1 M, 1.5 M and 2 M to investigate

infusion into the cell.

Any solution that comprises at least one substance which is oligosaccharide or polyhydric alcohol and allows the cellular uptake of the nucleic acid by bringing the hypertonic solution, the nucleic acid and cells into contact with each other can be used as the hypertonic solution of the present invention. Specifically, it is preferred that the hypertonic solution of the present invention should comprise a culture solution, a buffer solution and/or serum. It is more preferred that the hypertonic solution of the present invention should comprise all of a culture solution, a buffer solution and serum.

In this context, the culture solution used may be any culture solution suitable for each cell. The culture solution is exemplified by RPMI1640 (Invitrogen), DULBECCO'S MODIFIED EAGLE MEDIA (Invitrogen), F-10 Nutrient Mixture (Invitrogen), F-12 Nutrient Mixture (Invitrogen), Iscove's Modified Dulbecco's Media (Invitrogen) or MINIMUM ESSENTIAL MEDIA (Invitrogen).

The buffer solution is exemplified by a HEPES buffer solution (Sigma), a TRIS buffer solution (Sigma), or a PBS buffer solution (Invitrogen). The concentration of the buffer solution in the hypertonic solution may be any molarity. The molarity includes a range of preferably 0 M to 1 M, more preferably 1 mM to 100 mM, most preferably 1 mM to 10 mM.

The serum is exemplified by fetal bovine serum, bovine serum, calf serum or equine serum. The concentration of the serum in the hypertonic solution may be any concentration. The concentration includes a 5 range of preferably 0 to 20% (v/v), more preferably 5 to 10% (v/v).

Total molarity of the whole hypertonic solution of the present invention may be within a range of isotonic to 3 M.

10 The cell used in the method of nucleic acid infusion of the present invention is not limited by applied cell types in terms of the principle of the method. That is, as shown in Examples, the method of nucleic acid infusion of the present invention has 15 allowed nucleic acid infusion into a variety of cell types that are hardly infused with a nucleic acid heretofore. Thus, the method of nucleic acid infusion of the present invention can be applied to any cell type without limitations.

20 Specifically, the method of nucleic acid infusion can be applied to not only a cell to which usual nucleic acid infusion is readily applied, but also a hybridoma, a primary cultured hepatocyte, a primary cultured myoblast, a primary cultured muscle 25 cell, a primary cultured brown adipocyte precursor cell, a primary cultured brown adipocyte, a primary cultured white adipocyte precursor cell, a primary cultured white adipocyte or a primary cultured neuron

or the like.

Furthermore, the method of the present invention is not limited by the origins of cells in terms of the principle of the method and can be applied 5 to not only animal cells, but also plant cells, insect cells or nematode cells including protoplast forms thereof.

The method of nucleic acid infusion of the present invention is characterized by comprising (a) 10 bringing a nucleic acid, a hypertonic solution and cells into contact with each other and (b) lowering osmotic pressure of the above-described hypertonic solution after the step (a).

In this context, specifically, an isotonic 15 solution or a solution of lower osmotic pressure (hypotonic solution) and the cells may be brought into contact with each other for lowering the osmotic pressure of the hypertonic solution at the above-described step (b). It is more preferred to lower the 20 osmotic pressure by bringing the hypotonic solution and the cells into contact with each other. The specific composition of the hypotonic solution can include a hypotonic solution of a medium and water (preferably DEPC-treated water) mixed at a ratio of 6:4, or water 25 (e.g., distilled water and DEPC-treated water).

Next, a specific example of the method of nucleic acid infusion of the present invention will be illustrated.

At first, cells to be infused are cultured. The cells may be cultured with a culture solution suitable for each cell under usual conditions (37°C, 5% CO₂). The number (density) of cells may be the number 5 of cells at which each cell can favorably be grown.

A hypertonic solution and a hypotonic solution may appropriately be prepared to have the above-described composition of the hypertonic solution and the hypotonic solution of the present invention.

10 Nucleic acid infusion into the cells is performed as illustrated below. At first, a culture supernatant was removed from the cells, which are then brought into contact with a nucleic acid and the hypertonic solution. The contact may be performed by 15 any method by which the nucleic acid and the cells are eventually brought into contact with each other in the hypertonic solution. Specifically, the method is exemplified by a method in which the hypertonic solution containing the nucleic acid is prepared in 20 advance and then added to the above-described cells and a method in which the nucleic acid (a solution containing the nucleic acid) is supplemented initially with the cells and subsequently with the hypertonic solution.

25 The cells are then incubated for an appropriate period of time. The incubation time is preferably approximately 2 minutes to 20 minutes, more preferably approximately 10 minutes to 15 minutes.

After the hypertonic solution containing the nucleic acid is removed, the hypotonic solution is added.

Alternatively, water is added without the removal of the hypertonic solution, to render the solution

- 5 isotonic. The resulting solution may be incubated for an appropriate period of time, or otherwise after the removal of the added hypotonic solution, the hypotonic solution may be added again, followed by incubation for an appropriate period of time. The incubation time is
- 10 preferably approximately 2 minutes to 10 minutes. An incubation temperature after the addition of the above-described hypertonic solution and hypotonic solution is not particularly limited, as long as the resulting solution is kept in a liquid state at the temperature.
- 15 The incubation temperature includes a range of preferably 0°C to 42°C, more preferably room temperature to 37°C.

The hypotonic solution is then removed, and a usual growth medium is added. The nucleic acid

- 20 infusion is accomplished by these procedures. These procedures for infusion can be repeated.

The method of nucleic acid infusion of the present invention allows oligonucleotide infusion with higher efficiency than ever (50% or more, preferably 25 60% or more, more preferably 70% or more, even more preferably 80% or more efficiency). In addition, the method of the present invention allows efficient oligonucleotide infusion into cell types that are

hardly infused with a nucleic acid by conventional techniques. Since the method of the present invention does not employ adjuvants (such as liposomes and polyethylenimine) conventionally used, the method 5 overcomes the problems of conventional methods such as cytotoxicity and immunoadjuvant activity. Therefore, the present invention makes it possible to efficiently infuse oligonucleotide into not only adherent cell lines but also primary cultured cells and lymphocytes 10 that are highly sensitive to cytotoxicity.

The present invention provides a reagent for nucleic acid infusion used in the above-described method of nucleic acid infusion of the present invention.

15 The reagent for nucleic acid infusion of the present invention is characterized by comprising as an ingredient at least one substance which is oligosaccharide or polyhydric alcohol.

20 The oligosaccharide and the polyhydric alcohol can be prepared by a usual approach conventionally known in the art.

In this context, concrete examples of the oligosaccharide include disaccharide. The disaccharide includes sucrose, maltose, lactose, trehalose, 25 levanbiose, chitobiose, vicianose, sambubiose, melibiose, epicelllobiose, turanose, rutinose or robinobiose, with sucrose, maltose or lactose preferred.

The above-described polyhydric alcohol includes diol, triol, polyol or sugar alcohol, with triol, polyol or sugar alcohol more preferred.

In this context, the diol includes a glycol
5 derivative. The glycol derivative is exemplified by ethylene glycol.

The triol includes a glycerol derivative.
The glycerol derivative includes glycerol.

The polyol includes a polyglycol derivative.
10 The polyglycol derivative includes polyethylene glycol.
The polyethylene glycol may have any molecular weight not greater than 2000. Specifically, the polyethylene glycol is exemplified by PEG200, PEG300, PEG400, PEG600, PEG1000, PEG1500, PEG1540 and
15 PEG2000.

The above-described sugar alcohol includes monosaccharide alcohol or oligosaccharide alcohol. The monosaccharide alcohol includes mannitol, sorbitol, xylitol or erythritol.

20 The above-described oligosaccharide alcohol includes disaccharide alcohol.

The disaccharide alcohol includes maltitol, lactitol or reduced palatinose.

The reagent for nucleic acid infusion of the
25 present invention may comprise at least one substance which is a variety of oligosaccharides or polyhydric alcohols listed above. Specifically, the reagent for nucleic acid infusion may comprise at least one

substance arbitrarily selected from among the substances listed above, for example, sucrose, maltose, lactose, trehalose, levanbiose, chitobiose, vicianose, sambubiose, melibiose, epicellobiose, turanose,

5 rutinose, robinobiose, ethylene glycol, glycerol, polyethylene glycol (PEG having a molecular weight of 2000 or less, e.g., PEG200, PEG300, PEG400, PEG600, PEG1000, PEG1500, PEG1540 and PEG2000), mannitol, sorbitol, xylitol, erythritol, maltitol, lactitol and

10 reduced palatinose. To be more specific, the reagent for nucleic acid infusion may comprise one substance, two substances, three substances or four or more substances arbitrarily selected from among the substances listed above. In this case, the reagent for

15 nucleic acid infusion of the present invention may comprise each ingredient at any concentration, as long as the reagent for nucleic acid infusion of the present invention is prepared so that total molarity of the oligosaccharide and/or the polyhydric alcohol present

20 in a hypertonic solution prepared with the reagent for nucleic acid infusion falls within a range of 0.29 M to 3 M for allowing the cellular uptake of a nucleic acid when the hypertonic solution, the nucleic acid and cells are brought into contact with each other. The

25 total molarity of the oligosaccharide and/or the polyhydric alcohol in the hypertonic solution may be any molarity that falls within the above-described range and includes a range of preferably 0.35 M to 2.5

M, more preferably 0.35 M to 2.1 M, even more preferably 0.5 M to 2.1 M.

Although suitable molarity varies depending on cells, molarity suitable for a desired cell can 5 readily be determined, for example, by using three concentrations of 1 M, 1.5 M and 2 M to investigate infusion into the cell.

The reagent for nucleic acid infusion of the present invention may comprise at least one substance 10 which is oligosaccharide or polyhydric alcohol, as described above. The reagent for nucleic acid infusion may comprise only one substance or two ore more substances which are each oligosaccharide or polyhydric alcohol. When the reagent for nucleic acid infusion 15 comprises two or more substances which are each oligosaccharide or polyhydric alcohol, it is preferred that the reagent should comprise (1) at least one substance which is oligosaccharide or sugar alcohol and (2) at least one substance which is diol, triol or 20 polyol.

In this context, the oligosaccharide used includes disaccharide. The disaccharide includes sucrose, maltose, lactose, trehalose, levanbiose, chitobiose, vicianose, sambubiose, melibiose, 25 epicellobiose, turanose, rutinose or robinobiose, with sucrose, maltose or lactose preferred.

The above-described sugar alcohol includes monosaccharide alcohol or oligosaccharide alcohol.

The monosaccharide alcohol includes mannitol, sorbitol, xylitol or erythritol.

The above-described oligosaccharide alcohol includes disaccharide alcohol.

5 The disaccharide alcohol includes maltitol, lactitol or reduced palatinose.

The above-described diol includes a glycol derivative. The glycol derivative includes ethylene glycol.

10 The triol includes a glycerol derivative.

The glycerol derivative includes glycerol.

The polyol includes a polyglycol derivative. The polyglycol derivative includes polyethylene glycol (PEG).

15 The polyethylene glycol may have any molecular weight not greater than 2000. Specifically, the polyethylene glycol is exemplified by PEG200, PEG300, PEG400, PEG600, PEG1000, PEG1500, PEG1540 and PEG2000.

20 A preferred combination of (1) at least one substance which is oligosaccharide or sugar alcohol and (2) at least one substance which is diol, triol or polyol contained as an ingredient in the reagent for nucleic acid infusion of the present invention as
25 described above is exemplified by a combination between (1) at least one substance selected from sucrose, mannitol, sorbitol and xylitol and (2) at least one substance selected from PEG200, PEG300, PEG400, PEG600,

PEG1000, PEG1500, PEG1540, PEG2000 and glycerol.

More preferably, the reagent for nucleic acid infusion comprising the combination is exemplified by a reagent for nucleic acid infusion comprising (1) one substance which is oligosaccharide or sugar alcohol and (2) one substance which is diol, triol or polyol and specifically exemplified by a reagent for nucleic acid infusion comprising as an ingredient (1) sucrose, mannitol, sorbitol or xylitol and (2) PEG200, PEG300, PEG400, PEG600, PEG1000, PEG1500, PEG1540, PEG2000 or glycerol.

Such a reagent for nucleic acid infusion of the present invention comprising two or more substances which are each oligosaccharide or polyhydric alcohol may comprise each ingredient at any molarity, as long as the reagent for nucleic acid infusion of the present invention is prepared so that the oligosaccharide and/or the polyhydric alcohol in a hypertonic solution (i.e., the hypertonic solution of the present invention used in nucleic acid infusion) prepared with the reagent for nucleic acid infusion has a total molarity of 0.29 M to 3M for allowing the cellular uptake of a nucleic acid when the hypertonic solution, the nucleic acid, and cells are brought into contact with each other. The total molarity of the oligosaccharide and/or the polyhydric alcohol in the hypertonic solution includes a range of preferably 0.35 M to 2.5 M, more preferably 0.35 M to 2.1 M, even more

preferably 0.5 M to 2.1 M.

Although suitable molarity varies depending on cells, molarity suitable for a desired cell can readily be determined, for example, by using three 5 concentrations of 1 M, 1.5 M and 2 M to investigate infusion into the cell.

When the reagent for nucleic acid infusion of the present invention comprises two substances which are each oligosaccharide or polyhydric alcohol, the 10 molarity of each ingredient may be any value as described above, as long as total molarity of these two ingredients in the hypertonic solution falls within the above-described range. In this case, molarity of (1) one substance which is oligosaccharide or sugar alcohol 15 in the hypertonic solution can be exemplified by a range of 0.5 M to 2 M, and molarity of (2) one substance which is diol, triol or polyol in the hypertonic solution can be exemplified by 0.1 M.

When the reagent for nucleic acid infusion of 20 the present invention comprises only one substance which is oligosaccharide or polyhydric alcohol, it is preferred that the reagent should comprise one substance which is oligosaccharide or sugar alcohol.

In this context, concrete examples of the 25 oligosaccharide used include disaccharide, trisaccharide, tetrasaccharide, pentasaccharide or decasaccharide, with disaccharide preferred. The disaccharide includes sucrose, maltose, lactose,

trehalose, levanbiose, chitobiose, vicianose, sambubiose, melibiose, epicellobiose, turanose, rutinose or robinobiose, with sucrose, maltose or lactose preferred.

5 The above-described sugar alcohol includes monosaccharide alcohol or oligosaccharide alcohol.

 The monosaccharide alcohol includes mannitol, sorbitol, xylitol or erythritol.

10 The above-described oligosaccharide alcohol includes disaccharide alcohol.

 The disaccharide alcohol includes maltitol, lactitol or reduced palatinose.

15 It is preferred that the reagent for nucleic acid infusion of the present invention comprising only one substance which is oligosaccharide or sugar alcohol as described above should comprise sucrose, mannitol, sorbitol, xylitol or maltitol.

 Such a reagent for nucleic acid infusion of the present invention comprising only one substance which is oligosaccharide or polyhydric alcohol may comprise the substance (ingredient) at any molarity, as long as the oligosaccharide or the polyhydric alcohol present in a hypertonic solution (i.e., the hypertonic solution of the present invention used in nucleic acid infusion) prepared with the reagent for nucleic acid infusion of the present invention has a molarity of 0.29 M to 3M for allowing the cellular uptake of a nucleic acid when the hypertonic solution, the nucleic

acid, and cells are brought into contact with each other. The molarity of the oligosaccharide or the polyhydric alcohol in the hypertonic solution includes a range of preferably 0.35 M to 2.5 M, more preferably 5 0.35 M to 2.1 M, even more preferably 0.5 M to 2.1 M.

Although suitable molarity varies depending on cells, molarity suitable for a desired cell can readily be determined, for example, by using three concentrations of 1 M, 1.5 M and 2 M to investigate 10 infusion into the cell.

Each ingredient in the reagent for nucleic acid infusion of the present invention may be in the form of a solid or liquid. In this context, the solid form refers to waxy (e.g., wax) form, vaseline-like 15 form, flake-like form, crystallized form, powdery form, and the like. Alternatively, the liquid form refers to highly viscous form, low viscous form, nonviscous form, and the like.

When the reagent for nucleic acid infusion 20 comprises two or more ingredients (substances) belonging to the category of oligosaccharide or polyhydric alcohol, the ingredients may be each in independent solid or liquid form or may be formed into a solid or liquid mixture.

25 In this context, the independent solid form means that the ingredients are each individually solidified. The ingredients may be enclosed together in one container or enclosed in separate containers.

The above-described independent liquid form means that each of the ingredients is enclosed in a liquid state in a separate container.

The above-described solid mixture means that

5 the ingredients are mixed in a solid state, or are solidified with then mixed, and then enclosed in one container. The above-described liquid mixture means that the ingredients are mixed in a liquid state and enclosed in one container, or one of the ingredients in

10 a liquid state and the other of the ingredients in a solid state are mixed together and enclosed in one container.

Specifically, for example, (1) at least one substance which is oligosaccharide or sugar alcohol is

15 crystallized, and (2) at least one substance which is diol, triol or polyol is in waxy (wax) form. In this case, these two substances may be enclosed in one container. To be more specific, for example, (1)

20 sucrose in crystallized form and (2) polyethylene glycol in waxy (wax) form are enclosed in one container.

Each of the ingredients in solid form may be dissolved by a method (e.g., heating and dilution) suitable for each of them. The ingredients may be

25 prepared so that the hypertonic solution of the present invention finally exhibits the molarity shown above by the addition of a culture solution, a buffer solution and/or serum.

The reagent for nucleic acid infusion of the present invention as described above can be one of ingredients in a kit for nucleic acid infusion. The kit may consist of only the above-described reagent for 5 nucleic acid infusion of the present invention or may comprise the reagent for nucleic acid infusion of the present invention as well as other ingredients. Other ingredients in the kit include fluorescently labeled oligonucleotide and positive control siRNA.

10 The reagent and the kit for nucleic acid infusion of the present invention can be used as a nucleic acid infusion agent for allowing nucleic acid infusion.

15 The present invention provides a cell infused with a nucleic acid by the above-described method of nucleic acid infusion of the present invention.

As described above, the method of nucleic acid infusion of the present invention has allowed nucleic acid infusion into cells with high efficiency. 20 The method of the present invention has also allowed nucleic acid infusion into a variety of cells that are hardly infused with a nucleic acid heretofore. The present invention provides a cell infused with a nucleic acid by such a method of nucleic acid infusion 25 of the present invention.

The cell of the present invention may be any cell that is infused with a nucleic acid by the method of nucleic acid infusion of the present invention.

Examples of the cell include a cell obtained by infusing a nucleic acid into a macrophage-like cell line RAW264.7 cell, a hybridoma D011.10 cell, a primary cultured hepatocyte, a primary cultured myoblast, a
5 primary cultured muscle cell, a primary cultured adipocyte precursor cell, a primary cultured adipocyte or a primary cultured neuron by the method of nucleic acid infusion of the present invention.

Preferably, the cell includes a cell obtained
10 by infusing a nucleic acid into a cell that is hardly infused with a nucleic acid heretofore, that is, a macrophage-like cell line RAW264.7 cell, a hybridoma D011.10 cell, a primary cultured hepatocyte, a primary cultured myoblast, a primary cultured muscle cell, a
15 primary cultured adipocyte precursor cell, a primary cultured adipocyte or a primary cultured neuron by the method of nucleic acid infusion of the present invention.

These cells can be constructed by the above-
20 described method of nucleic acid infusion of the present invention.

The present invention provides RNA or a protein derived from a cell prepared from the cell of the present invention. The RNA or the protein derived
25 from the cell is prepared by a method already known in the art and can readily be prepared with reference to, for example, Molecular Cloning 3rd Edition (Cold Spring Harbor Press), Current Protocols in Molecular Biology

(John Wiley & Sons, Inc).

The present invention provides a method of functional analysis of a gene or a protein associated with an infused nucleic acid, characterized by using
5 the above-described cell of the present invention or the above-described RNA or protein of the present invention.

The method of nucleic acid infusion of the present invention allows every nucleic acid infusion as
10 described above, and can be used particularly preferably in the construction of a knockdown cell, which inhibits the expression and function of a target gene.

Examples of the functional analysis the uses
15 the knockdown cell include analysis in which an antisense nucleic acid, small interfering RNA or a decoy nucleic acid is infused into the cell and the production or function of a target gene or a protein encoded by the gene is inhibited to examine the
20 function of the gene in the cell.

It is preferred that the method of functional analysis of the present invention should conducted using a cell obtained by infusing a nucleic acid into a cell that is hardly infused with a nucleic acid
25 heretofore, that is, a macrophage-like cell line · RAW264.7 cell, a hybridoma D011.10 cell, a primary cultured hepatocyte, a primary cultured myoblast, a primary cultured muscle cell, a primary cultured

adipocyte precursor cell, a primary cultured adipocyte or a primary cultured neuron by the method of nucleic acid infusion of the present invention, or using RNA or a protein derived from the cell.

5

EXAMPLES

Hereinafter, the present invention will be described more specifically with reference to Examples. However, the present invention is not intended to be 10 limited to these Examples by any means.

Example 1

Infusion of oligonucleotide into murine macrophage-like cell line RAW264.7 cell using method of nucleic acid infusion of the present invention and 15 conventional methods

Murine macrophage-like cell line RAW264.7 cells were used to compare a method of nucleic acid infusion of the present invention that utilized osmotic shock (hereinafter, also referred to as an osmotic 20 transfection method) with conventional methods.

1-1) Infusion of oligonucleotide into murine macrophage-like cell line RAW264.7 cell by method of nucleic acid infusion of the present invention

1. Preparation of cell

25 RAW264.7 cells (ATCC No. TIB-71) were suspended in RPMI1640 (Invitrogen) containing 10% (v/v) fetal calf serum. The resulting cells were seeded at a density of 100000 cells/well into a 24-well plate

(Nalge Nunc) and cultured overnight under conditions of 37°C and 5% CO₂.

2. Preparation of hypertonic solution containing oligonucleotide

5 A hypertonic solution containing oligonucleotide was prepared to have the following composition: 10 μM FITC-labeled oligonucleotide (20-mer; 5'-GGA ACT TCC CTA AAG GGA GG-3'; SEQ ID NO.: 1), 1 M sucrose, 10% (w/v) polyethylene glycol 1000, 10 RPMI1640 (Invitrogen), 5% (v/v) fetal bovine serum, and 10 mM HEPES buffer solution (pH 7.4).

10 3. Preparation of hypotonic solution

A hypotonic solution was prepared to be a mixture solution of a RPMI1640 medium and DEPC-treated water (6:4).

15 4. Growth medium

RPMI1640 (Invitrogen) was supplemented with 10% (v/v) fetal bovine serum and 60 mg/L kanamycin sulfate to prepare a growth medium.

20 5. Infusion in 24-well plate

The RAW264.7 cells were seeded at 100000 cells/well and cultured overnight. The resulting culture supernatant was then removed, and 400 μl of the hypertonic solution containing oligonucleotide was added to the cells. Following incubation at 37°C for 10 minutes, the hypertonic solution containing oligonucleotide was removed. Immediately after the addition of 3 ml of the hypotonic solution, the

hypotonic solution was removed. Again, 3 ml of the hypotonic solution was added. Following incubation for 5 minutes, the hypotonic solution was removed, and 3 ml of the growth medium was added.

5 6. Observation of infusion efficiency

Infusion efficiency was observed by using a differential interference microscope Axiovert 135 (Carl Zeiss) equipped with a 20x objective and a super high-sensitivity CCD camera (Hamamatsu Photonics) to measure 10 excitation light from a fluorescence microscope system: a fluorescence microscopy apparatus for Axiovert 135 with an ultraviolet light source (Carl Zeiss), which passed through a FITC band pass filter. After 24 hours of infusion, FITC fluorescence was photographed with an 15 exposure for 2 seconds by the above-described fluorescence microscope system. The morphology of the cells was photographed with an exposure for 0.03 seconds under conditions of a visible light source and no filter by the same system.

20 1-2) Infusion of oligonucleotide into murine macrophage-like cell line RAW264.7 cell with Oligofectamine

1. Preparation of cell

RAW264.7 cells (ATCC No. TIB-71) were 25 suspended in RPMI1640 (Invitrogen) containing 10% (v/v) fetal calf serum. The resulting cells were seeded at a density of 100000 cells/well into a 24-well plate (Nalge Nunc) and cultured overnight under conditions of

37°C and 5% CO₂.

2. Infusion of oligonucleotide

An oligonucleotide/Oligofectamine mixture solution was prepared according to Invitrogen's recommendation manual. Specifically, 9 µl of Oligofectamine was suspended in 150 µl of OPTI-MEMI (Invitrogen) and left undisturbed at room temperature for 5 minutes. The resulting mixture was mixed with an equal amount of diluted oligonucleotide solution (a mixture solution of 9 µl of 20 µM FITC-labeled oligonucleotide and 150 µl of OPTI-MEMI) and left at room temperature for 20 minutes to obtain an oligonucleotide/Oligofectamine mixture solution. Then, the above-described cells prepared in the 24-well plate were washed with OPTI-MEMI to remove the culture supernatant. To the cells, 318 µl of the oligonucleotide/Oligofectamine mixture solution was gently added and left undisturbed at 37°C for 6 hours under 5% CO₂ to remove the resulting supernatant. The mixture was supplemented with 3 ml of the growth medium RPMI1640 (Invitrogen) containing 10% (v/v) fetal calf serum. After infusion at 37°C under 5% CO₂, culture was conducted for 24 hours.

3. Observation of infusion efficiency

Infusion efficiency was observed using the CCD camera as in the above-described Example 1-1).

1-3) Infusion of oligonucleotide into murine macrophage-like cell line RAW264.7 cell with

polyethylenimine1. Preparation of cell

RAW264.7 cells (ATCC No. TIB-71) were suspended in RPMI1640 (Invitrogen) containing 10% (v/v) fetal calf serum. The resulting cells were seeded at a density of 100000 cells/well into a 24-well plate (Nalge Nunc) and cultured overnight under conditions of 37°C and 5% CO₂.

2. Infusion of oligonucleotide

10 Polyethylenimine used was jetPEI (PolyPlus). An oligonucleotide/polyethylenimine mixture solution was prepared according to PolyPlus's recommendation manual. Specifically, 8 µl of 7.5 mM jetPEI was suspended in 192 µl of 150 mM NaCl aqueous solution
15 (Nacalai Tesque) and mixed with an equal amount of diluted oligonucleotide solution (40 µl of 20 µM FITC-labeled oligonucleotide and 160 µl of 150 mM NaCl aqueous solution) and left at room temperature for 15 minutes to obtain an oligonucleotide/polyethylenimine mixture solution. Then, 400 µl of the oligonucleotide/polyethylenimine mixture solution was gently added to the above-described cells prepared in the 24-well plate. After infusion at 37°C for 24 hours under 5% CO₂, culture was conducted for 24 hours.

25 3. Observation of infusion efficiency

Infusion efficiency was observed using the CCD camera as in the above-described Example 1-1).

1-4) Comparison of results of oligonucleotide infusion

among method of nucleic acid infusion of the present invention, Oligofectamine method and polyethylenimine method

The morphology of the cells, damage to the
5 cells and infusion efficiency after 24 hours of FITC-labeled oligonucleotide infusion were compared among the above-described method of nucleic acid infusion of the present invention, Oligofectamine method and polyethylenimine method. The result is shown in FIG.
10 1.

As a result, in the method of nucleic acid infusion of the present invention, the morphological change and damage of the cells were not observed, and oligonucleotide infusion was observed in 80% or more of
15 the cells in the visual field. On the other hand, in the Oligofectamine method and the polyethylenimine method, fluorescent oligonucleotide infusion was observed in almost all of the cells in the visual field. However, decrease in the number of cells due to
20 cell exfoliation during procedures for infusion was observed, while the morphological change of all of the cells in the bright field was observed and the great majority of the cells burst and died. These results have shown that unlike the Oligofectamine method and
25 the polyethylenimine method, the method of nucleic acid infusion of the present invention can infuse oligonucleotide without causing the morphological change and damage of cells.

Example 2

Infusion of TLR4 siRNA into RAW264.7 cell by method of nucleic acid infusion of the present invention and measurement of siRNA effect by

5 quantitative PCR1. Infusion of TLR4 siRNA into RAW264.7 cell by method of nucleic acid infusion of the present invention

TLR4 siRNA (double-stranded RNA with a sense sequence 5'-AUCCACAAGUCAAUCUCUUU-3'; SEQ ID NO: 2 and 10 an antisense sequence 5'-GAGAGAUUGACUUGUGGAUUU-3'; SEQ ID NO: 3) was synthesized by Proligo. Negative Control #1 siRNA was purchased from Ambion. The infusion of TLR4 siRNA and Negative Control #1 siRNA into RAW264.7 cells by the method of nucleic acid infusion of the 15 present invention was performed by the same approach as the above-described Example 1-1) after a hypertonic solution containing 10 µM TLR4 siRNA or Negative Control #1 siRNA was prepared in the same way as Example 1-1).

20 2. Collection of total RNA from infused cell and quantitative PCR

After 24 hours of infusion, total RNA was collected from the cells using an RNeasy kit (Qiagen). Primers specific to TLR4 mRNA (Forward Primer: 5'-
25 GGATGATGCCTCCCTGGC-3' (SEQ ID NO: 11) and Reverse Primer: 5'-GGGATTCAAGCTTCCTGGTG-3' (SEQ ID NO: 12)) and primers specific to GAPDH mRNA that served as a normalization control (Forward Primer: 5'-

AACTCGGCCCAACACT-3' (SEQ ID NO: 13) and Reverse Primer: 5'-TAGGCCCTCCTGTTATTATGG -3' (SEQ ID NO: 14)) were used to perform quantitative PCR as described below.

5 At first, TaqMan Reverse Transcription Reagents (Applied Biosystems) were used to synthesize cDNA with 1 µg of total RNA as a template according to the kit's protocol. The obtained cDNA derived from 50 ng of total RNA, which was used as a template, was
10 mixed with a specific primer set for detection and SYBR Green PCR Master Mix and RT-PCR Kit (Applied Biosystems) according to the kit's protocol to perform measurement with a real-time PCR system ABI7700 (Applied Biosystems). The rate of decrease in TLR4
15 mRNA was calculated with the expression level of GAPDH regarded as being equal between Negative Control #1 siRNA- and TLR4 siRNA-infused groups. The result is shown in FIG. 2.

As can be seen from the result, in the TLR4
20 siRNA-infused group, TLR4 mRNA disappeared 83% as compared with the Negative Control #1 siRNA-infused group.

These results have shown that the method of nucleic acid infusion of the present invention can
25 infuse not only fluorescently labeled oligonucleotide but also siRNA and that infused siRNA allows mRNA in cells to disappear with high efficiency.

Example 3

Infusion of TLR4 siRNA into RAW264.7 cell by
method of nucleic acid infusion of the present
invention and bioassay using knockdown cell

TLR4 is a receptor of LPS (Calbiochem) and
5 GLA-60 (WAKO CHEMICAL), and RAW264.7 cells are known to induce TNF α production by the stimulation of the cells with these compounds. Therefore, TLR4 siRNA was infused into RAW264.7 cells as described in Example 2. After 24 hours, the cells were scraped with a scraper
10 and suspended in a growth medium. The resulting cells were seeded at 10000 cells/well to a 96-well plate and cultured at 37°C under 5% CO₂. After 48 hours of the infusion of siRNA, the resulting culture supernatant was removed. Stimulation without or with 10 μ g/ml GLA-
15 60 or 10 ng/ml LPS for 8 hours was performed to collect the culture supernatant. Instead of TLR4 siRNA, Negative Control #1 siRNA (Sequitur) was infused as a control under the same conditions. After 48 hours, the resulting culture supernatant was removed. Stimulation
20 without or with 10 μ g/ml GLA-60 or 10 ng/ml LPS for 8 hours was performed to collect the culture supernatant.

TNF α contained in the culture supernatants of the TLR4 siRNA-infused cells and the control cells was quantified with a Mouse TNF- α AN'ALYZA ELISA kit (Cosmo Bio). The result is shown in FIG. 3. As can be seen from the result, in the TLR4 siRNA-infused cells, TNF α production decreased 45% by stimulation with LPS and 25 66% by stimulation with GLA-60 as compared with the

control cells.

These results have shown that the induction of TNF α production in response to stimulation with LPS or GLA-60 is observed in cells infused with Negative 5 Control siRNA by the method of nucleic acid infusion of the present invention as with usual cells whereas the induction of TNF α production in response to stimulation with these compounds is significantly inhibited in TLR4 siRNA-infused cells. Accordingly, these results have 10 indicated that siRNA infusion by the method of nucleic acid infusion of the present invention inhibits cell function, and more specifically, have indicated that the method of the present invention is useful for gene functional analysis such as the identification of a 15 receptor-ligand pair.

Example 4

Infusion of oligonucleotide into primary cultured rat hepatocyte by method of nucleic acid infusion of the present invention

20 1. Preparation of cell

A primary cultured rat hepatocyte was purchased from Hokudo. Specifically, the cells were received with them seeded at a density of 100000 cell/well in a 24-well plate (Nalge Nunc). Immediately 25 after the receipt of the cells, the original medium was replaced with an accompanying growth medium, and the cells were cultured overnight under conditions of 37°C and 5% CO₂.

2. Preparation of hypertonic solution containing oligonucleotide

A hypertonic solution containing oligonucleotide was prepared to have the following composition: 10 µM FITC-labeled oligonucleotide (20-mer; 5'-GGA ACT TCC CTA AAG GGA GG-3'; SEQ ID NO: 1), 1 M sucrose, 10% (w/v) polyethylene glycol 1000, RPMI1640 (Invitrogen), 5% (v/v) fetal bovine serum, and 10 mM HEPES buffer solution (pH 7.4).

10 3. Preparation of hypotonic solution

A hypotonic solution was prepared to be a mixture solution of a RPMI1640 medium and DEPC-treated water (6:4).

4. Growth medium

15 A medium included with a culture kit manufactured by Hokudo was used.

5. Infusion in 24-well plate

The culture supernatant of the hepatocytes was removed, and 400 µl of the hypertonic solution containing oligonucleotide was added to the cells. Following incubation at room temperature for 3 minutes, the hypertonic solution containing oligonucleotide was removed. Immediately after the addition of 3 ml of the hypotonic solution, the hypotonic solution was removed. Again, 3 ml of the hypotonic solution was added. Following incubation at room temperature for 5 minutes, the hypotonic solution was removed, and 3 ml of the growth medium was added.

6. Observation of infusion efficiency

Infusion efficiency was observed by using a differential interference microscope Axiovert 135 (Carl Zeiss) equipped with a 20x objective and a super high-sensitivity CCD camera (Hamamatsu Photonics) to measure excitation light from a fluorescence microscope system: a fluorescence microscopy apparatus for Axiovert 135 with an ultraviolet light source (Carl Zeiss), which passed through a FITC band pass filter. After 24 hours of infusion, FITC fluorescence was photographed with an exposure for 2 seconds by the above-described fluorescence microscope system. The morphology of the cells was photographed with an exposure for 0.03 seconds under conditions of a visible light source and no filter by the same system.

7. Result

Fluorescent oligonucleotide infusion was observed in almost all of the cells in the visual field. Moreover, morphological change and cell death were not observed in the cells in the bright field.

Example 5

Infusion of oligonucleotide into primary cultured rat myoblast by method of nucleic acid infusion of the present invention

25 1. Preparation of cell

A primary cultured rat myoblast was purchased from Hokudo. Specifically, the cells were received with them seeded at a density of 100000 cell/well in a

24-well plate (Nalge Nunc). Immediately after the receipt of the cells, the original medium was replaced with an accompanying growth medium, and the cells were cultured overnight under conditions of 37°C and 5% CO₂.

5 2. Preparation of hypertonic solution containing oligonucleotide

A hypertonic solution containing oligonucleotide was prepared to have the following composition: 10 μM FITC-labeled oligonucleotide (20-
10 mer; 5'-GGA ACT TCC CTA AAG GGA GG-3'; SEQ ID NO: 1), 1 M sucrose, 10% (w/v) polyethylene glycol 1000, RPMI1640 (Invitrogen), 5% (v/v) fetal bovine serum, and 10 mM HEPES buffer solution (pH 7.4).

3. Preparation of hypotonic solution

15 A hypotonic solution was prepared to be a mixture solution of a RPMI1640 medium and DEPC-treated water (6:4).

4. Growth medium

A medium included with a culture kit
20 manufactured by Hokudo was used.

5. Infusion in 24-well plate

The culture supernatant of the myoblasts was removed, and 400 μl of the hypertonic solution containing oligonucleotide was added to the cells.

25 Following incubation at room temperature for 10 minutes, the hypertonic solution containing oligonucleotide was removed. Immediately after the addition of 3 ml of the hypotonic solution, the

hypotonic solution was removed. Again, 3 ml of the hypotonic solution was added. Following incubation at room temperature for 5 minutes, the hypotonic solution was removed, and 3 ml of the growth medium was added.

5 6. Observation of infusion efficiency

Infusion efficiency was observed by using a differential interference microscope Axiovert 135 (Carl Zeiss) equipped with a 20x objective and a super high-sensitivity CCD camera (Hamamatsu Photonics) to measure 10 excitation light from a fluorescence microscope system: a fluorescence microscopy apparatus for Axiovert 135 with an ultraviolet light source (Carl Zeiss), which passed through a FITC band pass filter. After 24 hours of infusion, FITC fluorescence was photographed with an 15 exposure for 2 seconds by the above-described fluorescence microscope system. The morphology of the cells was photographed with an exposure for 0.03 seconds under conditions of a visible light source and no filter by the same system.

20 7. Result

Fluorescent oligonucleotide infusion was observed in almost all of the cells in the visual field. Moreover, morphological change and cell death were not observed in the cells in the bright field.

25 Example 6

Infusion of oligonucleotide into primary cultured rat muscle cell by method of nucleic acid infusion of the present invention

1. Preparation of cell

A primary cultured rat myoblast was purchased from Hokudo. Specifically, the cells were received with them seeded at a density of 100000 cell/well in a 5 24-well plate (Nalge Nunc). Immediately after the receipt of the cells, the original medium was replaced with an accompanying growth medium, and the cells were cultured overnight under conditions of 37°C and 5% CO₂. Then, the resulting culture supernatant was removed, 10 and the medium was replaced with an accompanying myoblast differentiation medium. The cells were cultured for 5 days to obtain differentiated muscle cells.

2. Preparation of hypertonic solution containing
15 oligonucleotide

A hypertonic solution containing oligonucleotide was prepared to have the following composition: 10 μM FITC-labeled oligonucleotide (20-mer; 5'-GGA ACT TCC CTA AAG GGA GG-3'; SEQ ID NO: 1), 1 20 M sucrose, 10% (w/v) polyethylene glycol 1000, RPMI1640 (Invitrogen), 5% (v/v) fetal bovine serum, and 10 mM HEPES buffer solution (pH 7.4).

3. Preparation of hypotonic solution

A hypotonic solution was prepared to be a 25 mixture solution of a RPMI1640 medium and DEPC-treated water (6:4).

4. Growth medium

A medium included with a culture kit

manufactured by Hokudo was used.

5. Infusion in 24-well plate

The culture supernatant of the differentiated muscle cells was removed, and 400 µl of the hypertonic solution containing oligonucleotide was added to the cells. Following incubation at room temperature for 10 minutes, the hypertonic solution containing oligonucleotide was removed. Immediately after the addition of 3 ml of the hypotonic solution, the 10 hypotonic solution was removed. Again, 3 ml of the hypotonic solution was added. Following incubation at room temperature for 5 minutes, the hypotonic solution was removed, and 3 ml of the growth medium was added.

6. Observation of infusion efficiency

15 Infusion efficiency was observed by using a differential interference microscope Axiovert 135 (Carl Zeiss) equipped with a 20x objective and a super high-sensitivity CCD camera (Hamamatsu Photonics) to measure excitation light from a fluorescence microscope system:
20 a fluorescence microscopy apparatus for Axiovert 135 with an ultraviolet light source (Carl Zeiss), which passed through a FITC band pass filter. After 24 hours of infusion, FITC fluorescence was photographed with an exposure for 2 seconds by the above-described
25 fluorescence microscope system. The morphology of the cells was photographed with an exposure for 0.03 seconds under conditions of a visible light source and no filter by the same system.

7. Result

Fluorescent oligonucleotide infusion was observed in almost all of the cells in the visual field. Moreover, morphological change and cell death 5 were not observed in the cells in the bright field.

Example 7

Infusion of oligonucleotide into primary cultured rat adipocyte by method of nucleic acid infusion of the present invention

10 1. Preparation of cell

A primary cultured rat white adipocyte precursor cell was purchased from Hokudo. Specifically, the cells were received with them seeded at a density of 100000 cell/well in a 24-well plate 15 (Nalge Nunc). Immediately after the receipt of the cells, the original medium was replaced with an accompanying differentiation medium, and the cells were cultured to obtain differentiated white adipocytes.

2. Preparation of hypertonic solution containing 20 oligonucleotide

A hypertonic solution containing oligonucleotide was prepared to have the following composition: 10 μ M FITC-labeled oligonucleotide (20-mer; 5'-GGA ACT TCC CTA AAG GGA GG-3'; SEQ ID NO: 1), 1 25 M sucrose, 10% (w/v) polyethylene glycol 1000, RPMI1640 (Invitrogen), 5% (v/v) fetal bovine serum, and 10 mM HEPES buffer solution (pH 7.4).

3. Preparation of hypotonic solution

A hypotonic solution was prepared to be a mixture solution of a RPMI1640 medium and DEPC-treated water (6:4).

4. Growth medium

5 A medium included with a culture kit manufactured by Hokudo was used.

5. Infusion in 24-well plate

The culture supernatant of the differentiated adipocytes was removed, and 400 µl of the hypertonic 10 solution containing oligonucleotide was added to the cells. Following incubation at room temperature for 10 minutes, the hypertonic solution containing oligonucleotide was removed. Immediately after the addition of 3 ml of the hypotonic solution, the 15 hypotonic solution was removed. Again, 3 ml of the hypotonic solution was added. Following incubation at room temperature for 2 minutes, the hypotonic solution was removed, and 3 ml of the growth medium was added.

6. Observation of infusion efficiency

20 Infusion efficiency was observed by using a differential interference microscope Axiovert 135 (Carl Zeiss) equipped with a 20x objective and a super high-sensitivity CCD camera (Hamamatsu Photonics) to measure excitation light from a fluorescence microscope system: 25 a fluorescence microscopy apparatus for Axiovert 135 with an ultraviolet light source (Carl Zeiss), which passed through a FITC band pass filter. After 24 hours of infusion, FITC fluorescence was photographed with an

exposure for 2 seconds by the above-described fluorescence microscope system. The morphology of the cells was photographed with an exposure for 0.03 seconds under conditions of a visible light source and
5 no filter by the same system.

7. Result

Fluorescent oligonucleotide infusion was observed in almost all of the cells in the visual field. Moreover, morphological change and cell death
10 were not observed in the cells in the bright field.

Example 8

Infusion of oligonucleotide into primary cultured rat neuron by method of nucleic acid infusion of the present invention

15 1. Preparation of cell

A primary cultured rat neuron was purchased from Sumitomo Bakelite. Specifically, the cells were seeded at a density of 100000 cell/well in an accompanying 24-well plate (Sumitomo Bakelite). After
20 replacement with an accompanying medium, the cells were cultured to obtain neurons.

2. Preparation of hypertonic solution containing oligonucleotide

A hypertonic solution containing
25 oligonucleotide was prepared to have the following composition: 10 μM FITC-labeled oligonucleotide (20-mer; 5'-GGA ACT TCC CTA AAG GGA GG-3'; SEQ ID NO: 1), 1 M sucrose, 10% (w/v) polyethylene glycol 1000, RPMI1640

(Invitrogen), 5% (v/v) fetal bovine serum, and 10 mM HEPES buffer solution (pH 7.4).

3. Preparation of hypotonic solution

A hypotonic solution was prepared to be a mixture solution of a RPMI1640 medium and DEPC-treated water (6:4).

4. Growth medium

A medium included with a culture kit manufactured by Hokudo was used.

10 5. Infusion in 24-well plate

The culture supernatant of the neurons was removed, and 400 µl of the hypertonic solution containing oligonucleotide was added to the cells.

Following incubation at room temperature for 10 minutes, the hypertonic solution containing oligonucleotide was removed. Immediately after the addition of 3 ml of the hypotonic solution, the hypotonic solution was removed. Again, 3 ml of the hypotonic solution was added. Following incubation at room temperature for 2 minutes, the hypotonic solution was removed, and 3 ml of the growth medium was added.

15 6. Observation of infusion efficiency

Infusion efficiency was observed by using a differential interference microscope Axiovert 135 (Carl Zeiss) equipped with a 20x objective and a super high-sensitivity CCD camera (Hamamatsu Photonics) to measure excitation light from a fluorescence microscope system: a fluorescence microscopy apparatus for Axiovert 135

with an ultraviolet light source (Carl Zeiss), which passed through a FITC band pass filter. After 24 hours of infusion, FITC fluorescence was photographed with an exposure for 2 seconds by the above-described

5 fluorescence microscope system. The morphology of the cells was photographed with an exposure for 0.03 seconds under conditions of a visible light source and no filter by the same system.

7. Result

10 Fluorescent oligonucleotide infusion was observed in almost all of the cells in the visual field. Moreover, morphological change and cell death were not observed in the cells in the bright field.

Example 9

15 Infusion of oligonucleotide into primary cultured rat adipocyte precursor cell using method of nucleic acid infusion of the present invention and conventional methods

Primary cultured rat adipocyte precursor
20 cells were used to compare the method of nucleic acid infusion of the present invention with conventional methods.

9-1) Infusion of oligonucleotide into primary cultured rat adipocyte precursor cell by method of nucleic acid
25 infusion of the present invention

1. Preparation of cell

A primary cultured rat white adipocyte precursor cell was purchased from Hokudo.

Specifically, the cells were received with them seeded at a density of 100000 cell/well in a 24-well plate (Nalge Nunc). Immediately after the receipt of the cells, the original medium was replaced with an 5 accompanying growth medium, and the cells were cultured.

2. Preparation of hypertonic solution containing oligonucleotide

A hypertonic solution containing 10 oligonucleotide was prepared to have the following composition: 10 µM FITC-labeled oligonucleotide (20-mer; 5'-GGA ACT TCC CTA AAG GGA GG-3'; SEQ ID NO: 1), 1 M sucrose, 10% (w/v) polyethylene glycol 1000, RPMI1640 (Invitrogen), 5% (v/v) fetal bovine serum, and 10 mM 15 HEPES buffer solution (pH 7.4).

3. Preparation of hypotonic solution

A hypotonic solution was prepared to be a mixture solution of a RPMI1640 medium and DEPC-treated water (6:4).

20 4. Growth medium

A medium included with a culture kit manufactured by Hokudo was used.

5. Infusion in 24-well plate

The culture supernatant of the white 25 adipocyte precursor cells was removed, and 400 µl of the hypertonic solution containing oligonucleotide was added to the cells. Following incubation at room temperature for 10 minutes, the hypertonic solution

containing oligonucleotide was removed. Immediately after the addition of 3 ml of the hypotonic solution, the hypotonic solution was removed. Again, 3 ml of the hypotonic solution was added. Following incubation at 5 room temperature for 2 minutes, the hypotonic solution was removed, and 3 ml of the growth medium was added.

6. Observation of infusion efficiency

Infusion efficiency was observed by using a differential interference microscope Axiovert 135 (Carl Zeiss) equipped with a 20x objective and a super high-sensitivity CCD camera (Hamamatsu Photonics) to measure excitation light from a fluorescence microscope system: a fluorescence microscopy apparatus for Axiovert 135 with an ultraviolet light source (Carl Zeiss), which 10 passed through a FITC band pass filter. After 24 hours of infusion, FITC fluorescence was photographed with an exposure for 2 seconds by the above-described 15 fluorescence microscope system. The morphology of the cells was photographed with an exposure for 0.03 seconds under conditions of a visible light source and no filter by the same system.

9-2) Infusion of oligonucleotide into primary cultured rat adipocyte precursor cell with Oligofectamine

1. Preparation of cell

25 A primary cultured rat white adipocyte precursor cell was purchased from Hokudo. Specifically, the cells were received with them seeded at a density of 100000 cell/well in a 24-well plate

(Nalge Nunc). Immediately after the receipt of the cells, the original medium was replaced with an accompanying growth medium, and the cells were cultured.

5 2. Infusion of oligonucleotide

An oligonucleotide/Oligofectamine mixture solution was prepared according to Invitrogen's recommendation manual. Specifically, 9 μ l of Oligofectamine was suspended in 150 μ l of OPTI-MEMI (Invitrogen) and left undisturbed at room temperature for 5 minutes. The resulting mixture was mixed with an equal amount of diluted oligonucleotide solution (a mixture solution of 9 μ l of 20 μ M FITC-labeled oligonucleotide and 150 μ l of OPTI-MEMI) and left at room temperature for 20 minutes to obtain an oligonucleotide/Oligofectamine mixture solution. Then, the above-described cells prepared in the 24-well plate were washed with OPTI-MEMI to remove the culture supernatant. To the cells, 318 μ l of the oligonucleotide/Oligofectamine mixture solution was gently added and left undisturbed at 37°C for 6 hours under 5%CO₂ to remove the resulting supernatant. The mixture was supplemented with 3 ml of the growth medium. After infusion at 37°C under 5% CO₂, culture was conducted for 24 hours.

20 3. Observation of infusion efficiency

Infusion efficiency was observed using the CCD camera as in the above-described Example 9-1).

9-3) Infusion of oligonucleotide into primary cultured rat adipocyte precursor cell with Lipofectamine 2000

1. Preparation of cell

A primary cultured rat white adipocyte precursor cell was purchased from Hokudo.
5 Specifically, the cells were received with them seeded at a density of 100000 cell/well in a 24-well plate (Nalge Nunc). Immediately after the receipt of the cells, the original medium was replaced with an 10 accompanying growth medium, and the cells were cultured.

2. Infusion of oligonucleotide

An oligonucleotide/Lipofectamine 2000 mixture solution was prepared according to Invitrogen's 15 recommendation manual. Specifically, 3 µl of Lipofectamine 2000 was suspended in 150 µl of OPTI-MEMI (Invitrogen) and left undisturbed at room temperature for 5 minutes. The resulting mixture was mixed with an equal amount of diluted oligonucleotide solution (a 20 mixture solution of 3 µl of 20 µM FITC-labeled oligonucleotide and 150 µl of OPTI-MEMI) and left at room temperature for 20 minutes to obtain an oligonucleotide/Lipofectamine 2000 mixture solution. Then, the above-described cells prepared in the 24-well 25 plate were washed with OPTI-MEMI to remove the culture supernatant. To the cells, 306 µl of the oligonucleotide/Lipofectamine 2000 mixture solution was gently added and left undisturbed at 37°C for 6 hours

under 5%CO₂ to remove the resulting supernatant. The mixture was supplemented with 3 ml of the growth medium. After infusion at 37°C under 5% CO₂, culture was conducted for 24 hours.

5 3. Observation of infusion efficiency

Infusion efficiency was observed using the CCD camera as in the above-described Example 9-1).

9-4) Comparison of results of oligonucleotide infusion into primary cultured rat adipocyte precursor cell

10 among method of nucleic acid infusion of the present invention, Oligofectamine method and Lipofectamine 2000 method

The morphology of the cells, damage to the cells and infusion efficiency after 24 hours of FITC-labeled oligonucleotide infusion were compared among the above-described method of nucleic acid infusion of the present invention, Oligofectamine method and Lipofectamine method.

As a result, in the method of nucleic acid infusion of the present invention, the morphological change and damage of the cells were not observed, and oligonucleotide infusion was observed in approximately 80% of the cells in the visual field. On the other hand, in the Oligofectamine method and the 25 Lipofectamine method, FITC-labeled oligonucleotide infusion was not observed in the great majority of the cells in the visual field. These results have shown that unlike the Oligofectamine method and the

Lipofectamine method, the method of nucleic acid infusion of the present invention can also infuse oligonucleotide into primary cultured rat adipocyte precursor cells without causing the morphological
5 change and damage of cells.

Example 10

Investigation on effect of sucrose concentration in hypertonic solution on infusion efficiency

10 While the composition of the hypertonic solution was the same as Example 1-1) except for sucrose, change in infusion efficiency was examined at varying sucrose concentrations: 0.5 M, 0.75M and 1 M.

1. Preparation of cell

15 RAW264.7 cells (ATCC No. TIB-71) were suspended in RPMI1640 (Invitrogen) containing 10% (v/v) fetal calf serum. The resulting cells were seeded at a density of 100000 cells/well into a 24-well plate (Nalge Nunc) and cultured overnight under conditions of
20 37°C and 5% CO₂.

2. Preparation of hypertonic solution containing oligonucleotide

A hypertonic solution containing oligonucleotide was prepared to have the following
25 composition: 10 μM FITC-labeled oligonucleotide (20-mer; 5'-GGA ACT TCC CTA AAG GGA GG-3'; SEQ ID NO: 1), 0.5 M, 0.75M or 1 M sucrose, 10% (w/v) polyethylene glycol 1000, RPMI1640 (Invitrogen), 5% (v/v) fetal

bovine serum, and 10 mM HEPES buffer solution (pH 7.4).

3. Preparation of hypotonic solution

A hypotonic solution was prepared to be a mixture solution of a RPMI1640 medium and DEPC-treated water (6:4).

4. Growth medium

RPMI1640 (Invitrogen) was supplemented with 10% (v/v) fetal bovine serum and 60 mg/L kanamycin sulfate to prepare a growth medium.

10 5. Infusion in 24-well plate

The RAW264.7 cells were seeded at 100000 cells/well and cultured overnight. The resulting culture supernatant was then removed, and 400 µl of the hypertonic solution containing oligonucleotide was added to the cells. Following incubation at room temperature for 10 minutes, the hypertonic solution containing oligonucleotide was removed. Immediately after the addition of 3 ml of the hypotonic solution, the hypotonic solution was removed. Again, 3 ml of the 15 hypotonic solution was added. Following incubation at room temperature for 2 minutes, the hypotonic solution was removed, and 3 ml of the growth medium was added.

20 6. Observation of infusion efficiency

Infusion efficiency was observed by using a differential interference microscope Axiovert 135 (Carl Zeiss) equipped with a 20x objective and a super high-sensitivity CCD camera (Hamamatsu Photonics) to measure excitation light from a fluorescence microscope system:

a fluorescence microscopy apparatus for Axiovert 135 with an ultraviolet light source (Carl Zeiss), which passed through a FITC band pass filter. After 24 hours of infusion, FITC fluorescence was photographed with an exposure for 2 seconds by the above-described fluorescence microscope system. The morphology of the cells was photographed with an exposure for 0.03 seconds under conditions of a visible light source and no filter by the same system.

10 7. Result

When the hypertonic solution containing 0.75 M or 1 M sucrose was used, fluorescent oligonucleotide infusion was observed in almost all of the cells in the visual field. When the hypertonic solution containing 0.5 M sucrose was used, fluorescent oligonucleotide infusion into the cells was observed although the number of cells infused with oligonucleotide was slightly lower than those for the hypertonic solution containing 0.75 M or 1 M sucrose. When the hypertonic solution of any of the sucrose concentrations was used, morphological change and cell death were not observed in the cells in the bright field. These results have shown that a more preferable sucrose concentration in a hypertonic solution is 0.5 M to 1 M.

25 Example 11

Investigation on effect of type of sugar in hypertonic solution on infusion efficiency

While the molarity of the solute in the

hypertonic solution was the same as Example 1-1), change in infusion efficiency was examined with various types of sugars: sucrose, mannitol, sorbitol and xylitol.

5 1. Preparation of cell

RAW264.7 cells (ATCC No. TIB-71) were suspended in RPMI1640 (Invitrogen) containing 10% (v/v) fetal calf serum. The resulting cells were seeded at a density of 100000 cells/well into a 24-well plate 10 (Nalge Nunc) and cultured overnight under conditions of 37°C and 5% CO₂.

2. Preparation of hypertonic solution containing oligonucleotide

A hypertonic solution containing 15 oligonucleotide was prepared to have the following composition: 10 μM FITC-labeled oligonucleotide (20-mer; 5'-GGA ACT TCC CTA AAG GGA GG-3'; SEQ ID NO: 1), 1 M sugar (sucrose, mannitol, sorbitol or xylitol), 10% (w/v) polyethylene glycol 1000, RPMI1640 (Invitrogen), 20 5% (v/v) fetal bovine serum, and 10 mM HEPES buffer solution (pH 7.4).

3. Preparation of hypotonic solution

A hypotonic solution was prepared to be a mixture solution of a RPMI1640 medium and DEPC-treated 25 water (6:4).

4. Growth medium

RPMI1640 (Invitrogen) was supplemented with 10% (v/v) fetal bovine serum and 60 mg/L kanamycin

sulfate to prepare a growth medium.

5. Infusion in 24-well plate

The RAW264.7 cells were seeded at 100000 cells/well and cultured overnight. The resulting culture supernatant was then removed, and 400 µl of the hypertonic solution containing oligonucleotide was added to the cells. Following incubation at room temperature for 10 minutes, the hypertonic solution containing oligonucleotide was removed. Immediately after the addition of 3 ml of the hypotonic solution, the hypotonic solution was removed. Again, 3 ml of the hypotonic solution was added. Following incubation at room temperature for 2 minutes, the hypotonic solution was removed, and 3 ml of the growth medium was added.

15 6. Observation of infusion efficiency

Infusion efficiency was observed by using a differential interference microscope Axiovert 135 (Carl Zeiss) equipped with a 20x objective and a super high-sensitivity CCD camera (Hamamatsu Photonics) to measure excitation light from a fluorescence microscope system: a fluorescence microscopy apparatus for Axiovert 135 with an ultraviolet light source (Carl Zeiss), which passed through a FITC band pass filter. After 24 hours of infusion, FITC fluorescence was photographed with an exposure for 2 seconds by the above-described fluorescence microscope system. The morphology of the cells was photographed with an exposure for 0.03 seconds under conditions of a visible light source and

no filter by the same system.

7. Result

No difference in infusion efficiency was observed among the sugars, sucrose, mannitol, sorbitol 5 and xylitol. These results have shown that any sugar can be used as a solute in a hypertonic solution.

Example 12

Investigation on effect of type of PEG in hypertonic solution on infusion efficiency

10 While the molarity of the solute in the hypertonic solution was the same as Example 1-1), change in infusion efficiency was examined with various types of PEGs: PEG200, PEG300, PEG400, PEG600, PEG1000, PEG1500, PEG1540 and PEG2000.

15 1. Preparation of cell

RAW264.7 cells (ATCC No. TIB-71) were suspended in RPMI1640 (Invitrogen) containing 10% (v/v) fetal calf serum. The resulting cells were seeded at a density of 100000 cells/well into a 24-well plate 20 (Nalge Nunc) and cultured overnight under conditions of 37°C and 5% CO₂.

2. Preparation of hypertonic solution containing oligonucleotide

A hypertonic solution containing 25 oligonucleotide was prepared to have the following composition: 10 μM FITC-labeled oligonucleotide (20-mer; 5'-GGA ACT TCC CTA AAG GGA GG-3'; SEQ ID NO: 1), 1 M sucrose, 10% (w/v) (=100 mM) polyethylene glycol 1000

or equal molarity of PEG (PEG200, PEG300, PEG400, PEG600, PEG1000, PEG1500, PEG1540 or PEG2000), RPMI1640 (Invitrogen), 5% (v/v) fetal bovine serum, and 10 mM HEPES buffer solution (pH 7.4).

5 3. Preparation of hypotonic solution

A hypotonic solution was prepared to be a mixture solution of a RPMI1640 medium and DEPC-treated water (6:4).

4. Growth medium

10 RPMI1640 (Invitrogen) was supplemented with 10% (v/v) fetal bovine serum and 60 mg/L kanamycin sulfate to prepare a growth medium.

5. Infusion in 24-well plate

The RAW264.7 cells were seeded at 100000 cells/well and cultured overnight. The resulting culture supernatant was then removed, and 400 µl of the hypertonic solution containing oligonucleotide was added to the cells. Following incubation at room temperature for 10 minutes, the hypertonic solution containing oligonucleotide was removed. Immediately after the addition of 3 ml of the hypotonic solution, the hypotonic solution was removed. Again, 3 ml of the hypotonic solution was added. Following incubation at room temperature for 2 minutes, the hypotonic solution was removed, and 3 ml of the growth medium was added.

25 6. Observation of infusion efficiency

Infusion efficiency was observed by using a differential interference microscope Axiovert 135 (Carl

Zeiss) equipped with a 20x objective and a super high-sensitivity CCD camera (Hamamatsu Photonics) to measure excitation light from a fluorescence microscope system: a fluorescence microscopy apparatus for Axiovert 135
5 with an ultraviolet light source (Carl Zeiss), which passed through a FITC band pass filter. After 24 hours of infusion, FITC fluorescence was photographed with an exposure for 2 seconds by the above-described fluorescence microscope system. The morphology of the
10 cells was photographed with an exposure for 0.03 seconds under conditions of a visible light source and no filter by the same system.

7. Result

No difference in infusion efficiency was
15 observed among the degrees of polymerization (average molecular weights) of PEGs. These results have shown that PEG with any degree of polymerization (average molecular weight) can be used as a solute in a hypertonic solution.

20 Example 13

Investigation on effect of substitution of glycerol for PEG in hypertonic solution on infusion efficiency

While the molarity of the solute in the
25 hypertonic solution was the same as Example 1-1), change in infusion efficiency was examined with equal molarity of glycerol substituted for 10% (w/v) (=100 mM) PEG1000.

1. Preparation of cell

RAW264.7 cells (ATCC No. TIB-71) were suspended in RPMI1640 (Invitrogen) containing 10% (v/v) fetal calf serum. The resulting cells were seeded at a density of 100000 cells/well into a 24-well plate (Nalge Nunc) and cultured overnight under conditions of 37°C and 5% CO₂.

2. Preparation of hypertonic solution containing oligonucleotide

10 A hypertonic solution containing oligonucleotide was prepared to have the following composition: 10 µM FITC-labeled oligonucleotide (20-mer; 5'-GGA ACT TCC CTA AAG GGA GG-3'; SEQ ID NO: 1), 1 M sucrose, 10% (w/v) (=100 mM) polyethylene glycol 1000 15 or 100 mM glycerol, RPMI1640 (Invitrogen), 5% (v/v) fetal bovine serum, and 10 mM HEPES buffer solution (pH 7.4).

3. Preparation of hypotonic solution

A hypotonic solution was prepared to be a mixture solution of a RPMI1640 medium and DEPC-treated water (6:4).

4. Growth medium

RPMI1640 (Invitrogen) was supplemented with 10% (v/v) fetal bovine serum and 60 mg/L kanamycin 25 sulfate to prepare a growth medium.

5. Infusion in 24-well plate

The RAW264.7 cells were seeded at 100000 cells/well and cultured overnight. The resulting

culture supernatant was then removed, and 400 μ l of the hypertonic solution containing oligonucleotide was added to the cells. Following incubation at room temperature for 10 minutes, the hypertonic solution 5 containing oligonucleotide was removed. Immediately after the addition of 3 ml of the hypotonic solution, the hypotonic solution was removed. Again, 3 ml of the hypotonic solution was added. Following incubation at room temperature for 2 minutes, the hypotonic solution 10 was removed, and 3 ml of the growth medium was added.

6. Observation of infusion efficiency

Infusion efficiency was observed by using a differential interference microscope Axiovert 135 (Carl Zeiss) equipped with a 20x objective and a super high-sensitivity 15 CCD camera (Hamamatsu Photonics) to measure excitation light from a fluorescence microscope system: a fluorescence microscopy apparatus for Axiovert 135 with an ultraviolet light source (Carl Zeiss), which passed through a FITC band pass filter. After 24 hours of infusion, 20 FITC fluorescence was photographed with an exposure for 2 seconds by the above-described fluorescence microscope system. The morphology of the cells was photographed with an exposure for 0.03 seconds under conditions of a visible light source and 25 no filter by the same system.

7. Result

No difference in infusion efficiency was observed between PEG and glycerol used. These results

have shown that not only PEG but also glycerol can be used as a solute in a hypertonic solution.

Example 14

Investigation on effect of substitution of

- 5 equal molarity of sucrose or sugar alcohol for PEG and sucrose in hypertonic solution of Example 1-1) on infusion efficiency

While the molarity of the solute in the hypertonic solution was the same as Example 1-1),
10 change in infusion efficiency was examined with equal molarity of sucrose, sorbitol, mannitol or xylitol substituted for 10% (w/v) (=100 mM) PEG1000 and 1 M sucrose.

1. Preparation of cell

15 RAW264.7 cells (ATCC No. TIB-71) were suspended in RPMI1640 (Invitrogen) containing 10% (v/v) fetal calf serum. The resulting cells were seeded at a density of 100000 cells/well into a 24-well plate (Nalge Nunc) and cultured overnight under conditions of
20 37°C and 5% CO₂.

2. Preparation of hypertonic solution containing oligonucleotide

A hypertonic solution containing oligonucleotide was prepared to have the following
25 composition: 10 μM FITC-labeled oligonucleotide (20-mer; 5'-GGA ACT TCC CTA AAG GGA GG-3'; SEQ ID NO: 1), 1 M sucrose and 10% (w/v) (=100 mM) PEG1000 or 1.1 M sugar (sucrose, sorbitol, mannitol or xylitol),

RPMI1640 (Invitrogen), 5% (v/v) fetal bovine serum, and 10 mM HEPES buffer solution (pH 7.4).

3. Preparation of hypotonic solution

A hypotonic solution was prepared to be a mixture solution of a RPMI1640 medium and DEPC-treated water (6:4).

4. Growth medium

RPMI1640 (Invitrogen) was supplemented with 10% (v/v) fetal bovine serum and 60 mg/L kanamycin sulfate to prepare a growth medium.

5. Infusion in 24-well plate

The RAW264.7 cells were seeded at 100000 cells/well and cultured overnight. The resulting culture supernatant was then removed, and 400 µl of the hypertonic solution containing oligonucleotide was added to the cells. Following incubation at room temperature for 10 minutes, the hypertonic solution containing oligonucleotide was removed. Immediately after the addition of 3 ml of the hypotonic solution, 20 the hypotonic solution was removed. Again, 3 ml of the hypotonic solution was added. Following incubation at room temperature for 2 minutes, the hypotonic solution was removed, and 3 ml of the growth medium was added.

6. Observation of infusion efficiency

25 Infusion efficiency was observed by using a differential interference microscope Axiovert 135 (Carl Zeiss) equipped with a 20x objective and a super high-sensitivity CCD camera (Hamamatsu Photonics) to measure

excitation light from a fluorescence microscope system: a fluorescence microscopy apparatus for Axiovert 135 with an ultraviolet light source (Carl Zeiss), which passed through a FITC band pass filter. After 24 hours 5 of infusion, FITC fluorescence was photographed with an exposure for 2 seconds by the above-described fluorescence microscope system. The morphology of the cells was photographed with an exposure for 0.03 seconds under conditions of a visible light source and 10 no filter by the same system.

7. Result

As can be seen from the result, the use of a variety of simple sugar substances (sucrose, sorbitol, mannitol and xylitol) allowed oligonucleotide infusion 15 as with the use of a mixture of PEG and sucrose when the simple sugar substance was used at the same molarity (1.1 M) as that of the mixture of PEG and sucrose. These results have shown that not only a mixture of PEG and sucrose but also each simple 20 ingredient substance can be used as a solute in a hypertonic solution.

Example 15

Infusion of oligonucleotide into murine macrophage-like cell line RAW264.7 cell using method of nucleic acid infusion of the present invention

For examining infusion efficiency and cytotoxicity by the method of nucleic acid infusion of the present invention, a murine macrophage-like cell

line RAW264.7 cell was used to examine, by flow cytometry, the effect of FITC-labeled nucleic acid infusion and the presence or absence of cytotoxicity caused by infusion.

5 15-1) Infusion of FITC-labeled oligonucleotide into murine macrophage-like cell line RAW264.7 cell by method of nucleic acid infusion of the present invention

1. Preparation of cell

10 RAW264.7 cells were cultured in RPMI1640 (Invitrogen) containing 10% (v/v) fetal calf serum in a T-75 flask (IWAKI) under conditions of 37°C and 5% CO₂.

2. Preparation of hypertonic solution

A hypertonic solution was prepared to have
15 the following composition: 1.4 M sucrose, 100 mM (10%) PEG1000, RPMI1640 (Invitrogen), 5% (v/v) fetal bovine serum, and 10 mM HEPES buffer solution (pH 7.4).

3. Preparation of FITC-labeled oligonucleotide solution

20 A FITC-labeled oligonucleotide solution was prepared in the same way as Example 1.

4. Hypotonic solution

Otsuka distilled water (Otsuka Pharmaceutical) was used as a hypotonic solution.

25 5. Growth medium

RPMI1640 (Invitrogen) was supplemented with 10% (v/v) fetal bovine serum and 60 mg/L kanamycin sulfate to prepare a growth medium.

6. Infusion in state of suspension

Into each 2.0-ml tube, 500000 RAW264.7 cells were collected and centrifuged at 2000 rpm for 5 minutes with a high-speed tabletop centrifuge (Tomy Seiko) to remove the resulting culture supernatant. To the tube, 10 μ l of 1 mM FITC-labeled oligonucleotide solution and 190 μ l of the hypertonic solution were added. Following incubation at 37°C for 10 minutes, 1.1 ml of Otsuka distilled water (Otsuka Pharmaceutical) was added. Following incubation for 10 minutes, the resulting mixture was centrifuged at 2000 rpm for 5 minutes with a high-speed tabletop centrifuge (Tomy Seiko) to remove the resulting culture supernatant. The mixture was resuspended in 2 ml of the growth medium, which was then seeded to a 6-well plate (IWAKI).

15-2) Measurement of effect of FITC-labeled oligonucleotide infusion

A flow cytometer EPICS-XL II system was used to examine the effect of oligonucleotide infusion with the amount of fluorescence emitted from fluorescein as an indicator.

After 24 hours of infusion, the cells were collected and centrifuged at 2000 rpm for 5 minutes with a high-speed tabletop centrifuge (Tomy Seiko) to remove the resulting culture supernatant. The resulting cells were resuspended in 1 ml of the growth medium. The fluorescence intensity of the fluorescein

in the obtained cells was measured using the EPICS-XL II system (Beckman Coulter). Fig 4 (upper diagram) shows a result for control cells obtained by using an equal amount of DEPC-treated water instead of FITC-labeled oligonucleotide and the cells infused with FITC-labeled oligonucleotide by the method of infusion of the present invention.

As a result, it has been shown that the use of the method of infusion of the present invention can 10 efficiently infuse oligonucleotide into murine macrophage-like cell line RAW264.7 cells.

15-3) Measurement of cytotoxicity

A flow cytometer EPICS-XL II system was used to examine cytotoxicity during infusion treatment with 15 the amount of fluorescence emitted from 7-actinomycin D (7AAD), a reagent for staining dead cells, as an indicator.

After 24 hours of infusion treatment, the cells were collected and centrifuged at 2000 rpm for 5 20 minutes with a high-speed tabletop centrifuge (Tomy Seiko) to remove the resulting culture supernatant. The resulting cells were resuspended in 1 ml of the growth medium. After 10 µl of a 7AAD solution (Beckman Coulter) was added and incubated at room temperature 25 for 10 minutes, the fluorescence intensity of the 7AAD in the obtained cells was measured using the EPICS-XL II system (Beckman Coulter). Fig 4 (lower diagram) shows a result for control cells treated in the same

way using the growth medium instead of the hypertonic and hypotonic solutions and the cells subjected to infusion treatment by the method of infusion of the present invention.

5 As a result, it has been shown that the infusion treatment of the present invention does not exhibit cytotoxicity.

These results have demonstrated that the method of infusion of the present invention can 10 efficiently infuse a nucleic acid and does not cause cytotoxicity.

Example 16

Infusion of siRNA into murine macrophage-like cell line RAW264.7 cell using method of nucleic acid infusion of the present invention

Murine macrophage cell line RAW264.7 cells were used to investigate the effect of types of oligosaccharide and polyhydric alcohol contained in the hypertonic solution on siRNA infusion into cells.

20 16-1) Infusion of siRNA into murine macrophage-like cell line RAW264.7 cell by method of nucleic acid infusion of the present invention

1. Preparation of cell

RAW264.7 cells (ATCC No. TIB-71) were 25 cultured in RPMI1640 (Invitrogen) containing 10% (v/v) fetal calf serum in a T-75 flask (IWAKI) under conditions of 37°C and 5% CO₂.

2. Preparation of hypertonic solution

A hypertonic solution was prepared to have the following composition: 1.4 M oligosaccharide or polyhydric alcohol, RPMI1640 (Invitrogen), 5% (v/v) fetal bovine serum, and 10 mM HEPES buffer solution (pH 5 7.4). Oligosaccharide used was sucrose (disaccharide, Sigma), and polyhydric alcohol used was sorbitol (monosaccharide alcohol, Sigma), mannitol (monosaccharide alcohol, Sigma), xylitol (monosaccharide alcohol, Sigma), maltitol (disaccharide alcohol, Sigma) or glycerol (triol, Nacalai Tesque).

3. Preparation of siRNA solution

siRNA was purchased from Proligo Japan as a freeze-dried product after the annealing of double strands. This siRNA was dissolved in DEPC-treated water (Nacalai Tesque) to adjust its concentration to 1 mM. The siRNA used was as follows: 1 mM Negative Control siRNA (21-mer sense strand; 5'-CAAGCUGACCCUGAAGUUCAU-3'; SEQ ID NO: 4 and 21-mer antisense strand; 5'-GAACUUCAGGGUCAGCUUGUU-3'; SEQ ID NO: 5, Proligo Japan) and 1 mM GAPDH siRNA (21-mer sense strand; 5'-CCACGAGAAUAUGACAACUC-3; SEQ ID NO: 6 and 21-mer antisense strand; 5'-GUUGUCAUAUUCUCGUGGUU-3'; SEQ ID NO: 7).

4. Hypotonic solution

25 Otsuka distilled water (Otsuka Pharmaceutical) was used as a hypotonic solution.

5. Growth medium

RPMI1640 (Invitrogen) was supplemented with

10% (v/v) fetal bovine serum and 60 mg/L kanamycin sulfate to prepare a growth medium.

6. Infusion in state of suspension

Into each 2.0-ml tube, 100000 RAW264.7 cells were collected and centrifuged at 2000 rpm for 5 minutes with a high-speed tabletop centrifuge (Tomy Seiko) to remove the resulting culture supernatant. To the tube, 10 µl of 1 mM siRNA solution and 190 µl of each hypertonic solution were added. Following incubation at 37°C for 10 minutes, 1.1 ml of Otsuka distilled water (Otsuka Pharmaceutical) was added. Following incubation for 10 minutes, the resulting mixture was centrifuged at 2000 rpm for 5 minutes with a high-speed tabletop centrifuge (Tomy Seiko) to remove the resulting culture supernatant. The mixture was resuspended in 2 ml of the growth medium, which was then seeded to a 6-well plate (IWAKI).

16-2) Measurement of effect on siRNA infusion

The effect of types of oligosaccharide and polyhydric alcohol contained in the hypertonic solution on siRNA infusion into cells was examined with the efficiency of GAPDH siRNA-mediated knockdown of endogenous GAPDH mRNA as an indicator.

After 24 hours of infusion, total RNA was collected from the cells using an RNeasy kit (Qiagen). Primers specific to GAPDH mRNA (Forward Primer: 5'-TCACCACCATGGAGAAGGC-3' (SEQ ID NO: 15) and Reverse Primer: 5'-CTAACGCAGTTGGTGGTGCAG-3' (SEQ ID NO: 16)) and

primers specific to cyclophilin mRNA that served as a normalization control (Forward Primer: 5'-GTCAACCCACCGTGTCT-3' (SEQ ID NO: 17) and Reverse Primer: 5'-GAGGTACCTGCTGTCTTG-3' (SEQ ID NO: 18))
5 were used to perform quantitative PCR in the same way as Example 2. The rate of decrease in GAPDH mRNA was calculated with the expression level of cyclophilin regarded as being equal between Negative Control siRNA- and GAPDH siRNA-infused groups. The result is shown in
10 FIG. 5.

As can be seen from the result, in the GAPDH siRNA-infused group, GAPDH mRNA disappeared 80.2% for the group with the hypertonic solution containing sucrose, 68.5% for the group with the hypertonic 15 solution containing sorbitol, 75.6% for the group with the hypertonic solution containing mannitol, 74.7% for the group with the hypertonic solution containing xylitol, 69.8% for the group with the hypertonic solution containing maltitol and 38.2% for the group 20 with the hypertonic solution containing glycerol as compared with the Negative Control siRNA-infused group.

These results have shown that the use of any oligosaccharide and polyhydric alcohol as a solute in a hypertonic solution also allows the efficient infusion 25 of siRNA as with FITC-labeled oligonucleotide and that the infused siRNA exhibits knockdown activity. Because the individual use of a variety of oligosaccharides and polyhydric alcohols as a solute in addition to the

combined use of oligosaccharide and PEG allowed the efficient siRNA infusion, it has been confirmed that the effect of infusion depends on the contact between cells and a hypertonic solution.

5 Example 17

Infusion of siRNA into murine macrophage-like cell line RAW264.7 cell using method of nucleic acid infusion of the present invention

Murine macrophage cell line RAW264.7 cells
10 were used to investigate the effect of a combination of oligosaccharide and polyhydric alcohol contained in the hypertonic solution on siRNA infusion into cells.

17-1) Infusion of siRNA into murine macrophage-like cell line RAW264.7 cell by method of nucleic acid infusion of the present invention

1. Preparation of cell

RAW264.7 cells (ATCC No. TIB-71) were cultured in RPMI1640 (Invitrogen) containing 10% (v/v) fetal calf serum in a T-75 flask (IWAKI) under
20 conditions of 37°C and 5% CO₂.

2. Preparation of hypertonic solution

A hypertonic solution was prepared to have the following composition: 1.4 M oligosaccharide, 100 mM polyhydric alcohol, RPMI1640 (Invitrogen), 5% (v/v)
25 fetal bovine serum, and 10 mM HEPES buffer solution (pH 7.4). Oligosaccharide used was sucrose or sorbitol, and polyhydric alcohol used was polyethylene glycol 200 (PEG200), PEG300, PEG400, PEG600, PEG1000, PEG1500,

PEG1540 or PEG2000. Sucrose and sorbitol were purchased from Sigma, and PEGs were purchased from Nacalai Tesque.

3. Preparation of siRNA solution

5 siRNA was purchased from Proligo Japan as a freeze-dried product after the annealing of double strands. This siRNA was dissolved in DEPC-treated water (Nacalai Tesque) to adjust its concentration to 1 mM. The siRNA used was as follows: 1 mM Negative
10 Control siRNA (21-mer sense strand; 5'-CAAGCUGACCCUGAAGUUCAU-3'; SEQ ID NO: 4 and 21-mer antisense strand; 5'-GAACUUCAGGGUCAGCUUGUU-3'; SEQ ID NO: 5, Proligo Japan) and 1 mM GAPDH siRNA (21-mer sense strand; 5'-CCACGAGAAUAUGACAACUC-3; SEQ ID NO: 6
15 and 21-mer antisense strand; 5'-GUUGUCAUAUUCUCGUGGUU-3'; SEQ ID NO: 7).

4. Hypotonic solution

Otsuka distilled water (Otsuka Pharmaceutical) was used as a hypotonic solution.

20 5. Growth medium

RPMI1640 (Invitrogen) was supplemented with 10% (v/v) fetal bovine serum and 60 mg/L kanamycin sulfate to prepare a growth medium.

6. Infusion in state of suspension

25 Into each 2.0-ml tube, 100000 RAW264.7 cells were collected and centrifuged at 2000 rpm for 5 minutes with a high-speed tabletop centrifuge (Tomy Seiko) to remove the resulting culture supernatant. To

the tube, 10 µl of 1 mM siRNA solution and 190 µl of each hypertonic solution were added. Following incubation at 37°C for 10 minutes, 1.1 ml of Otsuka distilled water (Otsuka Pharmaceutical) was added.

5. Following incubation for 10 minutes, the resulting mixture was centrifuged at 2000 rpm for 5 minutes with a high-speed tabletop centrifuge (Tomy Seiko) to remove the resulting culture supernatant. The mixture was resuspended in 2 ml of the growth medium, which was
- 10 then seeded to a 6-well plate (IWAKI).

17-2) Measurement of effect on siRNA infusion

The effect of a combination of oligosaccharide and polyhydric alcohol contained in the hypertonic solution on siRNA infusion into cells was examined with the efficiency of GAPDH siRNA-mediated knockdown of endogenous GAPDH mRNA as an indicator.

After 24 hours of infusion, total RNA was collected from the cells using an RNeasy kit (Qiagen). Primers specific to GAPDH mRNA (Forward Primer: 5'-
20 TCACCACCATGGAGAAGGC-3' (SEQ ID NO: 15) and Reverse Primer: 5'-CTAACGCAGTTGGTGGTGCAG-3' (SEQ ID NO: 16)) and primers specific to cyclophilin mRNA that served as a normalization control (Forward Primer: 5'-
GTCAACCCCACCGTGTCT-3' (SEQ ID NO: 17) and Reverse
25 Primer: 5'-GAGGTACCTGCTGTCTTG-3' (SEQ ID NO: 18)) were used to perform quantitative PCR in the same way as Example 2. The rate of decrease in GAPDH mRNA was calculated with the expression level of cyclophilin

regarded as being equal between Negative Control siRNA- and GAPDH siRNA-infused groups. A result for the combination of sucrose and a variety of PEGs is shown in FIG. 6, and a result for the combination of sorbitol 5 and a variety of PEGs is shown in FIG. 7.

As can be seen from the result, in the combination of sucrose and a variety of PEGs, GAPDH mRNA disappeared 69.2% for the group with the hypertonic solution containing sucrose and PEG200, 10 77.9% for the group with the hypertonic solution containing sucrose and PEG300, 69.1% for the group with the hypertonic solution containing sucrose and PEG400, 72.9% for the group with the hypertonic solution containing sucrose and PEG600, 68.7% for the group with 15 the hypertonic solution containing sucrose and PEG1000 and 73.3% for the group with the hypertonic solution containing sucrose and PEG1500. As can also be seen from the result, in the combination of sorbitol and a variety of PEGs, GAPDH mRNA disappeared 65.0% for the 20 group with the hypertonic solution containing sorbitol and PEG200, 62.9% for the group with the hypertonic solution containing sorbitol and PEG300, 49.6% for the group with the hypertonic solution containing sorbitol and PEG400, 41.8% for the group with the hypertonic 25 solution containing sorbitol and PEG600, 70.5% for the group with the hypertonic solution containing sorbitol and PEG1000, 57.7% for the group with the hypertonic solution containing sorbitol and PEG1500, 37.8% for the

group with the hypertonic solution containing sorbitol and PEG1540 and 45.4% for the group with the hypertonic solution containing sorbitol and PEG2000.

These results have shown that the use of a
5 combination of oligosaccharide and polyhydric alcohol
as a solute in a hypertonic solution also allows the
efficient infusion of siRNA as with FITC-labeled
oligonucleotide and that the infused siRNA exhibits
knockdown activity. The use of a combination of sugar
10 alcohol and PEGs as a solute in addition to a
combination of oligosaccharide and PEGs allowed the
efficient siRNA infusion, and the infused siRNA
exhibited knockdown activity. Therefore, it has been
confirmed that various combinations of oligosaccharide
15 and polyhydric alcohol can be used as a solute in a
hypertonic solution.

Example 18

Infusion of morpholino antisense
oligonucleotide into murine T cell-like cell line
20 DO11.10 cell using method of nucleic acid infusion of
the present invention

For determining whether or not the method of
nucleic acid infusion of the present invention required
the electric charge of a nucleic acid, murine T cell-
25 like cell line DO11.10 cells were used to examine the
effect of infusion of morpholino antisense
oligonucleotide, a nucleic acid analog with no electric
charge.

18-1) Infusion of morpholino antisense oligonucleotide into murine hybridoma DO11.10 cell by method of nucleic acid infusion of the present invention

1. Preparation of cell

5 DO11.10 cells were cultured in RPMI1640 (Invitrogen) containing 10% (v/v) fetal calf serum in a T-75 flask (IWAKI) under conditions of 37°C and 5% CO₂.

2. Preparation of hypertonic solution

A hypertonic solution was prepared to have
10 the following composition: 2.0 M sucrose, 100 mM PEG1000, RPMI1640 (Invitrogen), 5% (v/v) fetal bovine serum, and 10 mM HEPES buffer solution (pH 7.4).

3. Preparation of morpholino antisense oligonucleotide solution

15 Fluorescein-labeled morpholino antisense oligonucleotide was purchased from Funakoshi as a freeze-dried product. This morpholino antisense oligonucleotide was dissolved in DEPC-treated water (Nacalai Tesque) to adjust its concentration to 1 mM.

20 The fluorescein-labeled morpholino antisense oligonucleotide used was as follows: 1 mM morpholino antisense (25-mer; 5'-CCTCTTACCTCAGTTACAATTATA-3'; SEQ ID NO: 8).

4. Hypotonic solution

25 Otsuka distilled water (Otsuka Pharmaceutical) was used as a hypotonic solution.

5. Growth medium

RPMI1640 (Invitrogen) was supplemented with

10% (v/v) fetal bovine serum and 60 mg/L kanamycin sulfate to prepare a growth medium.

6. Infusion in state of suspension

Into each 2.0-ml tube, 500000 D011.10 cells were collected and centrifuged at 2000 rpm for 5 minutes with a high-speed tabletop centrifuge (Tomy Seiko) to remove the resulting culture supernatant. To the tube, 10 µl of 1 mM morpholino antisense oligonucleotide solution and 190 µl of the hypertonic solution were added. Following incubation at 37°C for 10 minutes, 1.1 ml of Otsuka distilled water (Otsuka Pharmaceutical) was added. Following incubation for 10 minutes, the resulting mixture was centrifuged at 2000 rpm for 5 minutes with a high-speed tabletop centrifuge (Tomy Seiko) to remove the resulting culture supernatant. The mixture was resuspended in 2 ml of the growth medium, which was then seeded to a 6-well plate (IWAKI).

18-2) Measurement of effect of morpholino antisense oligonucleotide infusion

A flow cytometer EPICS-XL II system was used to examine the effect of morpholino antisense oligonucleotide infusion with the amount of fluorescence emitted from fluorescein as an indicator.

25 After 24 hours of infusion, the cells were collected and centrifuged at 2000 rpm for 5 minutes with a high-speed tabletop centrifuge (Tomy Seiko) to remove the resulting culture supernatant. The

resulting cells were resuspended in 1 ml of the growth medium. The fluorescence intensity of the fluorescein in the obtained cells was measured using the EPICS-XL II system (Beckman Coulter). Fig 8 (upper diagram) 5 shows a result for control cells obtained by using an equal amount of DEPC-treated water instead of morpholino antisense oligonucleotide and the cells infused with morpholino antisense oligonucleotide by the method of infusion of the present invention. FIG. 10 8 (lower diagram) shows a result of infusing FITC-labeled phosphorothioate oligodeoxynucleotide, a nucleic acid analog with an electric charge, instead of morpholino antisense oligonucleotide under the same conditions

15 As can be seen from the result, the use of the method of infusion of the present invention allowed the efficient infusion of, into cells, not only a nucleic acid with an electric charge but also morpholino antisense oligonucleotide, a nucleic acid 20 analog with no electric charge.

These results have shown that the effect of the method of infusion of the present invention dose not depend on the electric charge of a nucleic acid.

Example 19

25 Repetitive infusion of siRNA into murine hybridoma DO11.10 cell using method of nucleic acid infusion of the present invention

Murine hybridoma DO11.10 cells were used to

examine the effect of GAPDH siRNA repetitive infusion.

19-1) Infusion of siRNA into murine hybridoma DO11.10 cell by method of nucleic acid infusion of the present invention

5 1. Preparation of cell

DO11.10 cells were cultured in RPMI1640 (Invitrogen) containing 10% (v/v) fetal calf serum in a T-75 flask (IWAKI) under conditions of 37°C and 5% CO₂.

2. Preparation of hypertonic solution

10 A hypertonic solution was prepared to have the following composition: 2.0 M sucrose, 100 mM PEG1000, RPMI1640 (Invitrogen), 5% (v/v) fetal bovine serum, and 10 mM HEPES buffer solution (pH 7.4).

3. Preparation of siRNA solution

15 siRNA was purchased from Proligo Japan as a freeze-dried product after the annealing of double strands. This siRNA was dissolved in DEPC-treated water (Nacalai Tesque) to adjust its concentration to 1 mM. The siRNA used was as follows: 1 mM Negative
20 Control siRNA (21-mer sense strand; 5'-CAAGCUGACCCUGAAGUUCAU-3'; SEQ ID NO: 4 and 21-mer antisense strand; 5'-GAACUUCAGGGUCAGCUUGUU-3'; SEQ ID NO: 5, Proligo Japan) and 1 mM GAPDH siRNA (21-mer sense strand; 5'-CCACGAGAAUAUGACAACUC-3; SEQ ID NO: 6
25 and 21-mer antisense strand; 5'-GUUGUCAUAUUCUCGUGGUU-3'; SEQ ID NO: 7).

4. Hypotonic solution

Otsuka distilled water (Otsuka

Pharmaceutical) was used as a hypotonic solution.

5. Growth medium

RPMI1640 (Invitrogen) was supplemented with
10% (v/v) fetal bovine serum and 60 mg/L kanamycin
5 sulfate to prepare a growth medium.

6. Infusion in state of suspension

Into each 2.0-ml tube, 500000 DO11.10 cells
were collected and centrifuged at 2000 rpm for 5
minutes with a high-speed tabletop centrifuge (Tomy
10 Seiko) to remove the resulting culture supernatant. To
the tube, 10 μ l of 1 mM siRNA solution and 190 μ l of
the hypertonic solution were added. Following
incubation at 37°C for 15 minutes, 1.5 ml of Otsuka
distilled water (Otsuka Pharmaceutical) was added.
15 Following incubation for 10 minutes, the resulting
mixture was centrifuged at 2000 rpm for 5 minutes with
a high-speed tabletop centrifuge (Tomy Seiko) to remove
the resulting culture supernatant. The mixture was
resuspended in 2 ml of the growth medium, which was
20 then seeded to a 6-well plate (IWAKI). For repetitive
infusion, the cells were collected every 24 hours of
infusion, and reinfusion was performed under the same
conditions.

19-2) Measurement of effect on siRNA infusion

25 The effect of types of oligosaccharide and
polyhydric alcohol contained in the hypertonic solution
on siRNA infusion into cells was examined with the
efficiency of GAPDH siRNA-mediated knockdown of

endogenous GAPDH mRNA as an indicator.

Total RNA was collected from each of the cells subjected to single infusion or repetitive infusion after 24 hours, 48 hours and 72 hours of 5 infusion using an RNeasy kit (Qiagen). Primers specific to GAPDH mRNA (Forward Primer: 5'-TCACCACCATGGAGAAGGC-3' (SEQ ID NO: 15) and Reverse Primer: 5'-CTAACGAGTTGGTGGTGCAG-3' (SEQ ID NO: 16)) and primers specific to cyclophilin mRNA that served as a 10 normalization control (Forward Primer: 5'-GTCAACCCCACCGTGTCT-3' (SEQ ID NO: 17) and Reverse Primer: 5'-GAGGTACCTGCTGTCTTG-3' (SEQ ID NO: 18)) were used to perform quantitative PCR in the same way as Example 2. The rate of decrease in GAPDH mRNA was 15 calculated with the expression level of cyclophilin regarded as being equal between Negative Control siRNA- and GAPDH siRNA-infused groups. The result is shown in FIG. 9.

As can be seen from the result, in the group 20 subjected to GAPDH siRNA single infusion, GAPDH mRNA disappeared 72.3% after 24 hours of infusion, 48.4% after 48 hours of infusion and 28.4% after 72 hours of infusion as compared with the Negative Control siRNA-infused group, whereas in the group subjected to GAPDH 25 siRNA repetitive infusion, GAPDH mRNA disappeared 71.4% after 24 hours of infusion, 70.4% after 48 hours of infusion and 68.0% after 72 hours of infusion as compared with the Negative Control siRNA-infused group.

These results have shown that the method of infusion of the present invention can also efficiently infuse siRNA into murine hybridoma DO11.10 cells as with FITC-labeled oligonucleotide and that the infused 5 siRNA exhibits knockdown activity. These results have also shown that reduction in the effect of knockdown with the passage of time was observed in single infusion, while the effect of siRNA-mediated knockdown can be maintained for any period by performing 10 repetitive infusion.

Example 20

Infusion of siRNA into primary cultured differentiated rat adipocyte by method of nucleic acid infusion of the present invention

15 20-1) Infusion of siRNA into primary cultured differentiated rat adipocyte by method of nucleic acid infusion of the present invention

1. Preparation of cell

A primary cultured rat white adipocyte 20 precursor cell was purchased from Hokudo. Specifically, the cells were received with them seeded at a density of 100000 cell/well in a 24-well plate (Nalge Nunc). Immediately after the receipt of the cells, the original medium was replaced with an 25 accompanying differentiation medium, and the cells were cultured to obtain differentiated white adipocytes.

2. Preparation of hypertonic solution

A hypertonic solution was prepared to have

the following composition: 1.0 M sucrose, 100 mM PEG1000, RPMI1640 (Invitrogen), 5% (v/v) fetal bovine serum, and 10 mM HEPES buffer solution (pH 7.4).

3. Preparation of siRNA solution

5 siRNA was purchased from ProLigo Japan as a freeze-dried product after the annealing of double strands. This siRNA was dissolved in DEPC-treated water (Nacalai Tesque) to adjust its concentration to 1 mM. The siRNA used was as follows: 1 mM Negative
10 Control siRNA (21-mer sense strand; 5'-CAAGCUGACCCUGAAGUUCAU-3'; SEQ ID NO: 4 and 21-mer antisense strand; 5'-GAACUUCAGGGUCAGCUUGUU-3'; SEQ ID NO: 5, ProLigo Japan) and 1 mM GAPDH siRNA (21-mer sense strand; 5'-CCACGAGAAUAUGACAACUC-3; SEQ ID NO: 6
15 and 21-mer antisense strand; 5'-GUUGUCAUAUUCUCGUGGUU-3'; SEQ ID NO: 7).

3. Preparation of hypotonic solution

A hypotonic solution was prepared to be a mixture solution of a RPMI1640 medium and DEPC-treated
20 water (6:4).

4. Growth medium

A medium included with a culture kit manufactured by Hokudo was used.

5. Infusion in 24-well plate

25 The culture supernatant of the differentiated adipocytes was removed, and 200 µl of the hypertonic solution containing siRNA was added to the cells. Following incubation at room temperature for 10

minutes, the hypertonic solution containing siRNA was removed. After the addition of 3 ml of the hypotonic solution and incubation at room temperature for 10 minutes, the hypotonic solution was removed, and 3 ml
5 of the growth medium was added.

20-2) Measurement of effect on siRNA infusion

The effect of types of oligosaccharide and polyhydric alcohol contained in the hypertonic solution on siRNA infusion into cells was examined with the
10 efficiency of GAPDH siRNA-mediated knockdown of endogenous GAPDH mRNA as an indicator.

Total RNA was collected from each of the cells subjected to single infusion after 24 hours, 48 hours and 72 hours of infusion using an RNeasy kit
15 (Qiagen). Primers specific to GAPDH mRNA (Forward Primer: 5'-TCACCACCATGGAGAAGGC-3' (SEQ ID NO: 15) and Reverse Primer: 5'-CTAACGCAGTTGGTGGTGCAG-3' (SEQ ID NO: 16)) and primers specific to cyclophilin mRNA that served as a normalization control (Forward Primer: 5'-
20 GTCAACCCCCACCGTGTCT-3' (SEQ ID NO: 17) and Reverse Primer: 5'-GAGGTACCTGCTGTCTTG-3' (SEQ ID NO: 18)) were used to perform quantitative PCR in the same way as Example 2. The rate of decrease in GAPDH mRNA was calculated with the expression level of cyclophilin
25 regarded as being equal between Negative Control siRNA- and GAPDH siRNA-infused groups. The result is shown in FIG. 10.

As can be seen from the result, in the GAPDH

siRNA-infused group, GAPDH mRNA disappeared 71.7% after 24 hours of infusion, 56.6% after 48 hours of infusion and 24.3% after 72 hours of infusion as compared with the Negative Control siRNA-infused group.

5 These results have shown that the method of infusion of the present invention can also efficiently infuse siRNA into primary cultured differentiated adipocytes as with FITC-labeled oligonucleotide and that the infused siRNA exhibits knockdown activity.

10 Example 21

Repetitive infusion of siRNA into murine macrophage-like cell line RAW264.7 cell using method of nucleic acid infusion of the present invention

Murine macrophage-like cell line RAW264.7 15 cells were used to examine the effect of TLR2 siRNA repetitive infusion at a protein level.

21-1) Infusion of siRNA into murine macrophage-like cell line RAW264.7 cell by method of nucleic acid infusion of the present invention

20 1. Preparation of cell

RAW264.7 cells were cultured in RPMI1640 (Invitrogen) containing 10% (v/v) fetal calf serum in a T-75 flask (IWAKI) under conditions of 37°C and 5% CO₂.

2. Preparation of hypertonic solution

25 A hypertonic solution was prepared to have the following composition: 1.4 M sucrose, 100 mM PEG1000, RPMI1640 (Invitrogen), 5% (v/v) fetal bovine serum, and 10 mM HEPES buffer solution (pH 7.4).

3. Preparation of siRNA solution

siRNA was purchased from Proligo Japan as a freeze-dried product after the annealing of double strands. This siRNA was dissolved in DEPC-treated water (Nacalai Tesque) to adjust its concentration to 1 mM. The siRNA used was as follows: 1 mM Negative Control siRNA (21-mer sense strand; 5'-CAAGCUGACCCUGAAGUUCAU-3'; SEQ ID NO: 4 and 21-mer antisense strand; 5'-GAACUUCAGGGUCAGCUUGUU-3'; SEQ ID NO: 5, Proligo Japan) and 1 mM TLR2 siRNA (21-mer sense strand; 5'-GUCUAAAGUCGAUCCGCGACA-3; SEQ ID NO: 9 and 21-mer antisense strand; 5'-UCGCGGAUCGACUUUAGACUU-3'; SEQ ID NO: 10).

4. Hypotonic solution

Otsuka distilled water (Otsuka Pharmaceutical) was used as a hypotonic solution.

5. Growth medium

RPMI1640 (Invitrogen) was supplemented with 10% (v/v) fetal bovine serum and 60 mg/L kanamycin sulfate to prepare a growth medium.

6. Infusion in state of suspension

Into each 2.0-ml tube, 500000 RAW264.7 cells were collected and centrifuged at 2000 rpm for 5 minutes with a high-speed tabletop centrifuge (Tomy Seiko) to remove the resulting culture supernatant. To the tube, 10 µl of 1 mM siRNA solution and 190 µl of the hypertonic solution were added. Following incubation at 37°C for 10 minutes, 1.1 ml of Otsuka

distilled water (Otsuka Pharmaceutical) was added.

Following incubation for 10 minutes, the resulting mixture was centrifuged at 2000 rpm for 5 minutes with a high-speed tabletop centrifuge (Tomy Seiko) to remove

5 the resulting culture supernatant. The mixture was resuspended in 2 ml of the growth medium, which was then seeded to a 6-well plate (IWAKI). The cells were collected after 24 hours of initial infusion, and reinfusion was performed under the same conditions.

10 21-2) Measurement at protein level

The effect of TLR2 siRNA infusion on endogenous TLR2 protein knockdown was examined.

Whole cell lysate was prepared from each of the Negative Control siRNA- and TLR2 siRNA-infused 15 cells after 48 hours of initial infusion using 1% (w/v) SDS aqueous solution. Protein quantification was performed with a BCA kit (Pierce). After 40 µg of each sample was mixed with 1/4 volumes of 5x sample buffer, the resulting mixture was subjected to electrophoresis 20 with 4%/20% gradient gel PAG Mini "Daiichi" 4/20 (Daiichi Pure Chemicals). After electrophoresis, BioRad Western Blotting System (BioRad) was used to blot the protein to Immobilon PVDF Transfer Membrane (Millipore). The membrane was then dipped in BlockAce 25 (Dainippon Pharmaceutical) and blocked by overnight incubation at 4°C.

For TLR2 detection, the membrane was incubated overnight at 4°C in a solution of an anti-TLR2

Antibody (IMG-526, IMGENEX) diluted 1/250 (diluted with BlockAce), and then washed with TBST (Tris-buffered saline with 0.1% Tween 20). Subsequently, the membrane was incubated at room temperature for 2 hours in a 5 solution of a goat anti-rabbit IgG-AP conjugate (BIOSOURCE) diluted 1/1000, and then washed with TBST (Tris-buffered saline with 0.1% Tween 20), followed by the staining of the membrane with Alkaline Phosphatase Conjugate Substrate Kit (BioRad). For GAPDH detection, 10 the membrane was incubated overnight at 4°C in a solution of anti-GAPDH (V-18, SantaCruz) diluted 1/100, and then washed with TBST (Tris-buffered saline with 0.1% Tween 20). Subsequently, the membrane was incubated at room temperature for 2 hours in a solution 15 of a donkey anti-goat IgG-AP conjugate (CHEMICON) diluted 1/1000, and then washed with TBST (Tris-buffered saline with 0.1% Tween 20), followed by the staining of the membrane with Alkaline Phosphatase Conjugate Substrate Kit (BioRad). The result is shown 20 in FIG. 11.

As can be seen from the result, in the TLR2 siRNA-infused group, the TLR2 protein decreased as compared with the Negative Control siRNA-infused group.

These results have shown that the effect of 25 knockdown mediated by siRNA infused by the method of infusion of the present invention is observed at a protein level.

The results obtained in the above-described

Examples 1 to 21 have revealed the followings:

- 1) the method of nucleic acid infusion of the present invention has the higher efficiency of nucleic acid infusion than those of conventional methods;
- 5 2) unlike conventional methods, the method of nucleic acid infusion of the present invention allows nucleic acid infusion without causing the morphological change and damage of cells, and the infused nucleic acid effectively functions in the cell;
- 10 3) the method of nucleic acid infusion of the present invention allows efficient nucleic acid infusion into cells that are previously reported to be hardly infused with a nucleic acid (such as a macrophage-like cell line RAW264.7 cell, a murine hybridoma DO11.10 cell, a
- 15 primary cultured hepatocyte, a primary cultured myoblast, a primary cultured muscle cell, a primary cultured adipocyte precursor cell, a primary cultured adipocyte and a primary cultured neuron), and the method can be applied to any cell type without
- 20 limitations;
- 4) the method of nucleic acid infusion of the present invention can be used regardless of the presence or absence of the electric charge of a nucleic acid;
- 5) ingredients in the hypertonic solution used in the
- 25 method of nucleic acid infusion of the present invention include oligosaccharide and polyhydric alcohol, and specifically, any type of polyethylene glycol, glycerol, sucrose and sugar alcohol (such as

mannitol, sorbitol, xylitol) can be used;

- 6) preferred molarity of the whole solute in the hypertonic solution used in the method of nucleic acid infusion of the present invention is approximately 0.5
- 5 to 2.1 M; and
- 7) the method of nucleic acid infusion of the present invention can be used in repetitive infusion.

INDUSTRIAL APPLICABILITY

10 A novel method of nucleic acid infusion of the present invention allows nucleic acid infusion with higher efficiency than ever. In addition, the method of nucleic acid infusion of the present invention allows nucleic acid infusion without causing the

15 morphological change and damage of cells. Therefore, the infused nucleic acid can effectively function in the cell. The method of infusion of the present invention also allows efficient nucleic acid infusion into cells that are previously reported to be hardly

20 infused with a nucleic acid. Thus, the method of nucleic acid infusion of the present invention can be used quite effectively in gene functional analysis (e.g., gene functional analysis such as identification of a receptor-ligand pair) that targets every cell that

25 is conventionally unable to be analyzed.

Sequence Listing Free Text

SEQ ID NO: 1 is a base sequence of synthetic

oligonucleotide.

SEQ ID NO: 2 is a base sequence of synthetic oligonucleotide.

SEQ ID NO: 3 is a base sequence of synthetic
5 oligonucleotide.

SEQ ID NO: 4 is a base sequence of synthetic oligonucleotide.

SEQ ID NO: 5 is a base sequence of synthetic oligonucleotide.

10 SEQ ID NO: 6 is a base sequence of synthetic oligonucleotide.

SEQ ID NO: 7 is a base sequence of synthetic oligonucleotide.

15 SEQ ID NO: 8 is a base sequence of synthetic oligonucleotide.

SEQ ID NO: 9 is a base sequence of synthetic oligonucleotide.

SEQ ID NO: 10 is a base sequence of synthetic oligonucleotide.

20 SEQ ID NO: 11 is a base sequence of a PCR primer.

SEQ ID NO: 12 is a base sequence of a PCR primer.

25 SEQ ID NO: 13 is a base sequence of a PCR primer.

SEQ ID NO: 14 is a base sequence of a PCR primer.

SEQ ID NO: 15 is a base sequence of a PCR

primer.

SEQ ID NO: 16 is a base sequence of a PCR
primer.

SEQ ID NO: 17 is a base sequence of a PCR
5 primer.

SEQ ID NO: 18 is a base sequence of a PCR
primer.